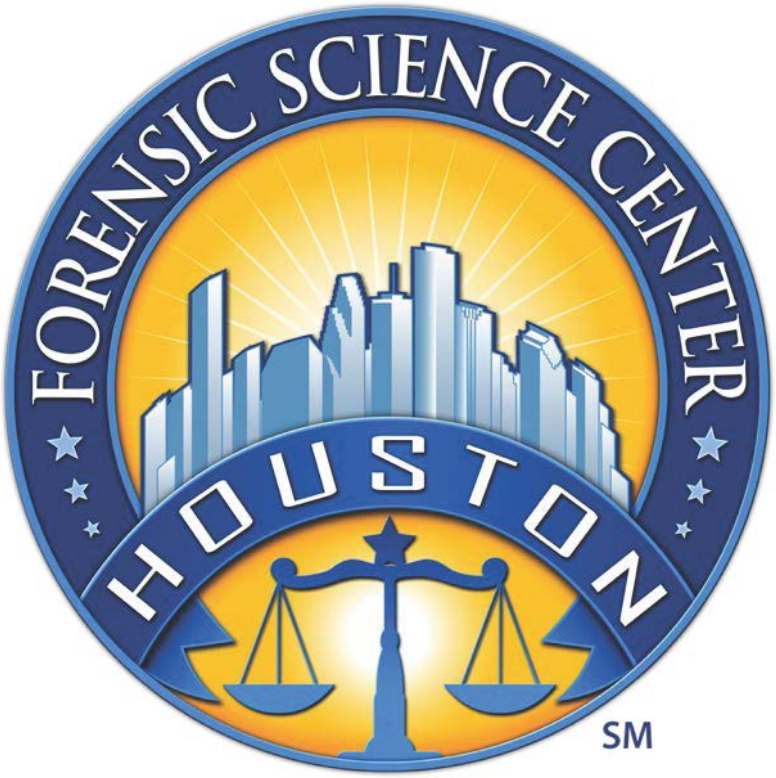


# Toxicology
# Analytical Manual

Forensic Analysis Division

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# Introduction

This Toxicology Standard Operating Procedures manual ("manual" or "SOP manual") is intended to consolidate the policies and procedures specific to the toxicology section of the Houston Forensic Science Center ("HFSC"). Any conflict between this document and any governing policies established by HFSC, such as the overall quality assurance system, shall be resolved in favor of the HFSC policy. This manual is part of an overall quality assurance system in the Houston Forensic Science Center. Any policies established in the governing quality system or in the policies established by the Houston Forensic Science Center will supersede any requirements stated in this manual. However, this document may add additional guidance that supplements what has already been established. This document is an amalgamation of multiple existing policies and procedures specific to the toxicology section. This document supersedes any existing policies and procedures that are not incorporated into this document.

# Safety

These procedures must be conducted in accordance with the existing Health and Safety Manual and the current Quality Assurance Manual. All biological samples shall be treated with universal precautions. Appropriate personal protective equipment should be worn at all times. Flammable liquids and vapors may cause eye, skin, and respiratory tract irritation. Derivatization reagents are toxic and must be handled in a chemical safety hood or well-ventilated area. Material Safety Data Sheets (MSDS) are available in the laboratory.

# Evidence Handling

1 **PURPOSE**
This document outlines the handling procedures of submitted evidence into the Toxicology Section.

2 **SCOPE**
This procedure is used for the handling and storage of evidence within the Toxicology Section.

3 **SUBMISSION OF EVIDENCE**
Evidence to be analyzed by the Toxicology Section is received from law enforcement agencies or the judicial system.

4 **STORAGE OF EVIDENCE**
Toxicology evidence is routinely stored in refrigerators within the toxicology section. Freezer storage is an acceptable alternative. All refrigerators and freezers in the toxicology section are monitored using TempAlert, which is further detailed in the Quality Manual, or equivalent system.

| | |
|---|---|
| Acceptable refrigerator temperature range: | > 0 - 10°C |
| Acceptable freezer temperature range: | ≤ 0°C |

If a refrigerator/freezer stops functioning and exceeds the acceptable temperature range, evidence will be moved to another functioning refrigerator/freezer and transfer documented.

5 **RECEIVING EVIDENCE**
**It is the responsibility of Toxicology personnel to maintain the integrity of evidence at all times while in their custody. All evidence must be protected from loss, cross-transfer, contamination, or deleterious change.**

5.1 Upon receipt of evidence into the Toxicology Section:

5.1.1 Toxicology personnel will examine evidence container(s) to ensure that proper seals, which are further detailed in the Quality Manual, are in place.

5.1.2 All evidence transfers must be documented electronically as part of the chain of custody and include comments pertaining to any evidence processing, excluding storage. If needed, a paper chain of custody will suffice using the HFSC Chain of Custody Form**.**

5.1.3 The outer-most container, *e.g.* a bag, envelope, or box containing specimens for a case, must be marked with a unique case identifier. This may be accomplished by adding a LIMS barcode label associated with the evidence to the container.

5.1.4 A submission form, or an electronic equivalent, must be filled out for all submitted evidence and contain the following information: agency case number, subject name(s), and description(s) of evidence. If date(s) of birth is used as an identifier on the evidence, the date of birth must be included in the submission information. If any of the above information is missing, the evidence will be returned and a rejection report will be issued.

## 6 ACCESSIONING

All specimens related to a case will be compared with the submission form and evidence packaging documentation for proper accessioning (inventory). The accessioner will mark all inner evidence packaging with unique case identifier and initials, as well as sub-itemize and mark specimens with the sub-item number, unique case identifier, date, and initials. This may also be accomplished by printing LIMS mini barcode labels which include the sub-item number, unique case identifier, date, and unique employee number.

### 6.1 Discrepancies

Fatal discrepancies will result in a report to the customer indicating "the evidence has been rejected for analysis due to [standard phrase highlighted in bold for each discrepancy]" or an equivalent statement, and the evidence being returned. All fatal discrepancies will be documented in the case record; minor discrepancies will be captured in the photographs.

#### 6.1.1 **Fatal discrepancies include**:

6.1.1.1 **Inconsistent subject name** (this includes instances where the name is missing on any pieces of evidence, when the name is not exactly the same throughout all pieces of evidence, or subject name between the evidence and submission information do not match)

6.1.1.2 **Date of birth of subject given on evidence contradicts date of birth given on submission information**

6.1.1.3 **Inconsistent agency case numbers** (this includes instances where the case number varies between any pieces of evidence or the case number between the evidence and submission information do not match)

6.1.1.4 **Specimens missing affixed labels or specimens with affixed labels missing pertinent information** (this includes at least two of the following: subject name, agency case number, or date of birth)

6.1.1.5 **Outer-most evidence container not being properly sealed**

6.1.1.6 **All specimens are compromised, *e.g.* leaking, cracked, or tampered container(s)**

6.1.1.7 **Inconsistent evidence descriptions between evidence received, submission form, and evidence documentation**

#### 6.1.2 Minor discrepancies

Minor discrepancies include any missing or contradicting information not pertaining to fatal discrepancies.

### 6.2 Photographs

Evidence must have representative images uploaded into LIMS. The naming scheme of Item# – Picture#, *e.g.* Item1 – 1, may be used. Pictures may include:

6.2.1 Outer-most container, including all sides

6.2.2 Inner plastic box

6.2.3 Blood vials in plastic tubing

6.2.4 Blood vials at all angles

6.2.5 Urine specimen containers at all angles

6.2.6 Any other exhibits

6.3 **Sub-itemizing**

A description of specimens within a case, along with the corresponding sub-item number, must be documented on the Evidence Description and Review Form (LAB-32) or an electronic equivalent. Evidence must be sub-itemized in LIMS and correspond with the documentation. For cases with multiple evidence items, separate evidence forms will be used for each item.

6.3.1 Common parent item descriptions in LIMS include: ONE TOXICOLOGY KIT, TWO BLOOD VIALS, ONE BLOOD VIAL, or ONE URINE SPECIMEN

6.3.2 Common sub-item descriptions include:

6.3.2.1 one color top tube

6.3.2.2 one color top tube with replacement top

6.3.2.3 one replacement top tube

6.3.2.4 one plastic container

6.3.3 For cases that have multiple color top tubes, the best suited one will be analyzed as follows: grey>lavender>pink>tan>royal blue (if it contains anticoagulant). The following color top tubes require discussion with manager/supervisor and client to decide the appropriateness of testing in the event they are the only type provided: gold or red/grey, orange, light green or green/grey, white, red, royal blue(if it contains a clot activator), green, light blue. Yellow top BD brand tubes will not be analyzed.

6.4 **Assignments/tasks**

Based on type of offense and type of evidence submitted, the following assignments/tasks will be added to LIMS, unless otherwise requested. For cases with multiple subjects associated with separate evidence items, an assignment/task will be added for each subject. Three types of reports may be issued: alcohol, negative screening, and confirmation.

6.4.1 DWI – Misdemeanor
Blood Specimens – ALC, if <0.10 → Drug Screen: EIA, if positive → Confirmation

6.4.2 DWI – Felony
Blood Specimens – ALC, if <0.10 → Drug Screen: EIA + BAN, if positive → Confirmation

6.4.3 DUID
Blood Specimens – ALC, if <0.10 → Drug Screen: EIA, if positive → Confirmation
Urine Specimen – Drug Screen: EIA + BAN, if positive → Confirmation

6.4.4 Vehicular Homicide or Death Due to Accident
Blood Specimens – ALC → Drug Screen: EIA + BAN, if positive → Confirmation

6.4.5 Sexual Assault (Toxicology Kit usually contains both blood and urine specimens)
Blood Specimens only – ALC → Drug Screen: EIA + BAN, if positive → Confirmation
Blood Specimens – ALC
Urine Specimen – Drug Screen: EIA + BAN, if positive → Confirmation

6.4.6 Other – Assignment will be made based on client request or consult

## 7 OUTSOURCED CASES

7.1 Evidence to be outsourced to an external laboratory must be processed in the following manner:

7.1.1 Verify correct evidence case number

7.1.2 Add a barcode label or manually transfer evidence custody in LIMS

7.1.3 Document outsourcing of evidence through chain of custody in LIMS

7.1.4 Verify the photograph of the evidence has been taken
7.1.5 Seal the evidence for shipment

7.2 Evidence that has been outsourced for testing and has been returned must be verified and documented prior to being sealed and returned to the submitting agency. This is accomplished by:
7.2.1 Verifying correct evidence case number
7.2.2 Adding a barcode label or manually transferring evidence custody in LIMS
7.2.3 Document receiving of evidence through chain of custody in LIMS
7.2.4 Photographing evidence
7.2.5 Sealing evidence

## 8 RETURNING OF EVIDENCE

8.1 All submitted items will be returned to submitting agency.
8.2 Before evidence is sealed, the contents will be checked for proper labeling and one or more pictures will be taken of the tested specimen to record the initials of analysts having opened the container to conduct analysis.
8.3 Outer evidence containers will be properly sealed and labeled with initials of the individual placing the seal on the item and date the seal was placed before returning evidence to the submitting agency. A part of the initials or date must extend over the edge of the seal onto the container.

# Technical and Administrative Review

1 **PURPOSE**

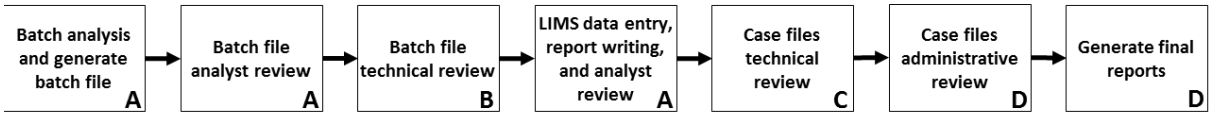
This document outlines the technical and administrative review process for batch analysis of human toxicology specimens.

2 **SCOPE**

This procedure is used to manage the technical and administrative review of toxicology documentation.

3 **REVIEW PROCESS**

Following batch casework analysis, a batch file will be created by the casework analyst conducting the analysis. The batch file will then be technically reviewed initially by the casework analyst and afterwards by another qualified analyst using the appropriate Batch Review Checklist. Upon successful completion of the batch technical review, the casework analyst will then enter case-related information into the Laboratory Information Management System (LIMS), resulting in case reports. Each case will then be reviewed by three qualified analysts using the appropriate Case File Review Checklist in the following order: casework analyst, technical reviewer, and administrative reviewer. Casework analyst review of each case file consists of both a technical and administrative review. Below is a schematic of the overall review process:




**A** = Casework analyst
**B** = Any qualified analyst not **A**
**C** = Any qualified analyst not **A**
**D** = Any qualified analyst not **A** or **C**

Any errors caught during technical or administrative review must be addressed before proceeding to the next task in the review process. If an error is caught during administrative review of a case file, the error must be corrected and the case file technically and administratively reviewed once more.

If the batch file is printed, the casework analyst will scan and store the documentation in the proper digital location following successful batch file technical review. If the batch file exists digitally, it will be moved, if not already, to the proper digital location. Printed or digital case-specific documentation will then be moved to each respective case record.

4 **BATCH FILE**

A batch file will be created, printed or digitally, by the casework analyst.

4.1 Batch files must include:

4.1.1 Batch Review Checklist
4.1.2 Work list
4.1.3 Sequence
4.1.4 Calibration/Controls
4.1.5 Data
4.1.6 Worksheet summarizing the data and detailing other information concerning testing

4.2 Batch files may include:
4.2.1 Method
4.2.2 Blood and urine batch report
4.2.3 Any corrective action documentation

## 5 CASE FILE

A case file is generated, printed or digitally, upon submission of evidence to the Houston Forensic Science Center. Each case file must include the following:

### 5.1 Examination Documentation

5.1.1 Data relevant to the case
5.1.2 Worksheet summarizing the results and detailing other information concerning testing

NOTE: For each report generated (e.g., alcohol, negative screening, and/or positive screening/confirmation), the appropriate Case File Review Checklist will include total number of examination pages associated with the report.

### 5.2 Administrative Documentation

5.2.1 Case File Review Checklist
5.2.2 Evidence Description and Review Form
5.2.3 Submission Form
5.2.4 Photographs of submitted evidence

5.2.5 Administrative documentation may include:
5.2.5.1 Report
5.2.5.2 Discovery Order
5.2.5.3 Correspondence (phone, email, and/or other types of communication)
5.2.5.4 Any recovery or corrective action documentation
5.2.5.5 Other documentation, *e.g.* outside agency forms

## 6 BATCH REVIEW CHECKLIST

Batch review checklists, which are part of the case record, detail the aspects of the batch file requiring inspection for technical accuracy. These have been developed for alcohol (LAB-70 ALC Batch Review Checklist), immunoassay screening (LAB-75 Immunoassay Batch Checklist), and GC/MS screening and/or confirmation (LAB-73 Batch Review Checklist – Drug Toxicology). Equivalent electronic checklists may also be used.

## 7 CASE FILE REVIEW CHECKLIST

Case file review checklists, which are part of the case record, detail the aspects of the case file requiring inspection for technical and administrative accuracy. These have been developed for both alcohol (LAB-71 ALC Case File Review Checklist) and screening/confirmation (LAB-74 Case File Review – Drug Toxicology). Equivalent electronic checklists may also be used.

# Preparation of Drug Free Matrix

1 **PURPOSE**

Preparation and preservation of drug-free matrices using sodium fluoride and/or sodium azide.

2 **SCOPE**

Drug-free matrices are used for the preparation of calibrators and controls for screening and confirmatory toxicology tests.

Drug-free blood containing sodium fluoride preservative and potassium oxalate as anticoagulant is purchased from a commercial vendor. Drug-free urine is prepared in-house and preserved using sodium fluoride and/or sodium azide. Other biological matrices may be prepared as needed.

3 **SAFETY/QUALITY ASSURANCE**

This procedure must be conducted in accordance with the Safety Manual, the Chemical Hygiene Plan, and the Quality Manual.

4 **REAGENTS**

4.1 Sodium fluoride (ACS grade or better)

4.2 Potassium oxalate (ACS grade or better)

5 **EQUIPMENT**

5.1 Stirrer

5.2 Glass media bottles

5.3 Stir bars

5.4 Top loading balance

5.5 Homogenizer (for preparation of tissues only)

6 **PROCEDURE**

6.1 Drug-Free Blood:

Drug-free blood containing sodium fluoride preservative (1%) and potassium oxalate (0.2%) as anticoagulant is purchased from a commercial vendor. If blood does not contain the preservative and anticoagulant, it can be prepared in-house at the specified concentrations. Store drug-free blood at 0-10°C (3 month expiration from date of receipt for toxicology testing purposes).

6.2 Drug-Free Urine for Immunoassay and GC/MS qualitative analyses:

6.2.1 Human urine from drug-free individuals is collected into a disposable plastic specimen cup or other collection containers and refrigerated.

6.2.2 Once approximately 500 mL of urine has been collected it should be pooled and tested by immunoassay and/or GCMS Screen to ensure it is drug free.

6.2.3 Verified drug-free urine shall be transferred into individual plastic containers each containing approximately 10 mL and frozen.

6.2.4 On day of use, thaw sufficient urine to complete assay and combine before use.

6.2.5 Alternatively, commercial drug-free human urine products can be used once they are demonstrated not to interfere with the analytical assays in service.

6.2.6 Store drug-free urine at <0°C (12 month expiration).

6.3 Quality Assurance/Quality Control

All drug-free matrices are appropriately tested using screening or confirmatory drug testing prior to use. Documentation is maintained in a retrievable format.

6.3.1 Drug-free blood or urine is evaluated in triplicate for each relevant assay as negative control samples (with internal standard if applicable). The samples should not trigger positive results or affect performance of the assays. When relevant, signal abundance after corrected by internal standard should be less than 10% of that of LOQ.

# Verification of Relative Concentrations of Stock Standards

1 **PURPOSE**

This procedure may be performed to compare a new stock standard solution with an old stock standard solution. The following experiment is done when required in order to verify the relative concentrations of the calibration and the control stock standards.

2 **SCOPE**

This SOP is applicable to all laboratory staff and this procedure is not routinely required to be performed unless specified in a protocol, method, or SOP.

3 **PROCEDURE**

This procedure may be performed when two separate stock standard solutions for a compound are used (one for the calibrators and another for the controls and/or validation standards), and the nature of the study requires a defined relative accuracy between the two stock solutions or the nature of the study requires a defined accuracy between the calibrators and controls/validation standards prepared from these stock solutions. It will be done before the preparation of batches of controls or validation standards that are to be used in a method validation or characterization. Once a method is established, correct preparation of calibrators and controls can be verified using the SOP "Preparation and Validation of Drug Standards, Spiking Solutions, Controls, and Reagents".

3.1 Each of the two stock standards are to be precisely diluted in triplicate in an appropriate solvent so that they may be analyzed directly by the instrumental technique that will be used for analysis of subject specimens. Each dilution is to be analyzed in duplicate, giving a total of 12 analyses.

3.1.1 For example, if the compound of interest is to be analyzed by GC-MS, dilutions will be prepared from each stock solution by diluting each stock solution with reconstitution solvent and then analyzing by GC-MS. Each dilution will be prepared in triplicate and injected in duplicate, giving a total of 12 injections. These dilutions must be prepared so that they are within the linear response range of the GC-MS instrument. A typical procedure would read:

3.1.2 Prepare triplicate dilutions of each of the 100 ng/µL stock standard solutions by adding 20 µL of the stock standards into conical tubes. For instance, using a 25 µL syringe, add 2.0 mL of freshly prepared reconstitution solvent to each 13mm x 100mm culture tube and vortex briefly to mix. Transfer to autosampler vials and analyze using the normal analytical conditions. Make duplicate injections of each dilution.

3.2 **Data Analysis**

Using the peak area or peak height response, determine the response factor of the stock solutions by dividing the average response of the duplicate injections for each stock solution by the solution concentration. Calculate the response factor ratio by dividing the response factors of one of the stock solutions by the response factor for the other stock solution. A ratio of 1.0 ± 0.03 gives confidence in the preparation of the stock solutions.

# Preparation and Validation of Drug Standards, Spiking Solutions, Controls and Reagents

## 1 PURPOSE

Organic solvents should be HPLC grade or higher and inorganic reagents (e.g. salts) should be ACS grade. Deionized water should be prepared using a Millipore Direct Q UV3 water system or from an equivalent source. Any internally prepared calibrators or controls may be purchased from an appropriate vendor in lieu of preparation in house

All drug standards, spiking solutions, and quality control preparations must be documented on LAB-68 Reagent Preparation Worksheet or equivalent and include key information regarding drug standard and chemical names, manufacturers, lot numbers for both manufacturer, and new solutions, verification dates, and by whom the solution was prepared. Reference drug standards, controls, and reagents used in the laboratory must be of sufficient quality for their intended use.

## 2 SCOPE

This procedure can be used for the qualitative or quantitative analysis of all toxicology specimens.

## 3 DEFINITIONS

3.1 Certified Reference Material (CRM): Drug standard purchased from an approved vendor which includes a certificate of analysis verifying the concentration.

3.2 Drug standard: any chemical other than the sample used in the preparation of standard solutions for calibrators, controls or internal reference. CRM should be used as drug standards whenever possible.

3.3 Calibration Sample: Analytical standard used to fix, set or check the graduations or scale of an analytical procedure

3.4 Internal Standard (IS): An analyte (generally of similar chemical structure to an analyte being measured) that is added, in a known concentration, to all samples (calibrators, QC's and unknowns) in an analytical method, and that functions as a reference marker for that sample, against which the analyte of interest can be measured.

3.5 Fortified Quality Control (QC) sample: A sample of similar matrix to the unknown case sample, which has been spiked with a predetermined amount of the analyte(s) of interest. QC samples can be prepared in-house or purchased from an approved Vendor.

3.6 Spiking Solution: Solution prepared by diluting drug standard to a pre-determined concentration and used to prepare calibration or QC Samples

3.7 Reagent: a chemical, chemical mixture or dilution of a chemical substance used in toxicological analysis.

## 4 DRUG STANDARDS – PURCHASING, STORAGE, AND EXPIRATION

4.1 Vendors should supply a Certificate of Analysis that contains specific free and formula molecular weights, purity, storage conditions, solubility, and a lot number. Information may include an expiration or re-test date.

4.2 Drug standards purchased as liquids in sealed ampoules expire on the date indicated by the manufacturer. Solid drug standards expire on the date indicated by the manufacturer.

4.3 Once a drug standard solution is prepared, transferred, or diluted, it expires within one year unless specified otherwise in the SOP.

4.4 A drug standard may not be used for quantitative analysis if the drug control or standard in question is beyond the expiration date. Expired drug standards may be used only for qualitative analysis beyond their expiration date if they are verified prior to use. GC/MS is the preferred technique for qualitative verification purposes. These verified standards are clearly identified as "Qualitative only."

4.5 An Internal Standard prepared from a solid reference material or sealed ampoules may be used beyond the expiration date provided by the manufacturer, provided that:

4.5.1 It is re-verified prior to use; and

4.5.2 Documented on LAB-68 Reagent Preparation Worksheet or equivalent; and

4.5.3 Provides results within the acceptance criteria specified in the standard operating procedure or by the manufacturer.

4.6 Biological controls may be used beyond the manufacturer's expiration date provided that the above criteria are met.

4.7 Expired reagents should be discarded or clearly labeled not for casework.

## 5 CALIBRATION SAMPLES

Assay calibration must be performed as validated and described in the analytical method.

5.1 **QC Samples**: Types of controls, in order of preference:

5.1.1 Commercial controls

5.1.2 In-house controls prepared in bulk

5.1.3 Controls prepared at the time of analysis using a "spiking solution"

5.2 **Preparation of spiking solutions/calibration samples, internal standard solutions, controls, and reagents**

5.2.1 Standards must be made and stored in accordance with the SOP. An equivalent procedure may be used if it is documented on the appropriate preparation log.

5.2.2 In-house prepared controls and/or spiking solutions should be prepared from a different manufacturer than the CRM used to prepare calibration samples.

5.2.3 If a drug standard is not available from a different manufacturer then a different lot from the same manufacturer can be used. If a drug standard is not available from a different manufacturer or a different lot from the same manufacturer, different ampoules should be used to make separate stock solutions for controls/spiking solutions and calibration samples.

5.2.4 QC samples must be prepared at a different time and by a different analyst than the solutions used for calibration samples.

5.2.5 Information regarding preparation must be documented using the appropriate form or an equivalent form or method, for example:

5.2.5.1 LAB-27: Multi-Component Working Stock/Standard Preparation Log;

5.2.5.2 LAB-28: Toxicology Single Component Working Stock/Standard Preparation Log;

5.2.5.3 LAB-68: Reagent Preparation Worksheet

5.2.6 All solutions/samples must be labeled accordingly. The label must contain at a minimum the

solution name and concentration, solution preparation date, initials of the preparer, and expiration date.

5.2.7 Spiking solutions, QC Samples, and IS solutions must be verified to ensure that the solutions and QC samples been correctly prepared before being used in casework. Upon verification, the verifying analyst shall note the date of verification and initial that date.

5.2.8 The concentration of purchased control material must be verified prior to being used with casework. Upon verification, the verifying analyst shall note the date of verification and initial that date. Verification run must be documented and data kept in a retrievable format in the laboratory.

5.3 **Validation of new lots of spiking solutions/calibration samples**

5.3.1 **Validation batch must include:**

5.3.1.1 Current calibrator set

5.3.1.2 New calibrator set

5.3.1.3 QC Samples

5.3.2 **Evaluation of new spiking solutions/calibration samples**

5.3.2.1 Calculate batch as normal, using current calibrator set as “calibrators”

5.3.2.2 Treat the new calibrators and quality controls as unknowns and determine their calculated values

5.3.2.3 If possible, repeat steps using new calibrator set as “calibrators”

5.3.2.4 Open the Calibrator Validation Excel Spreadsheet Template

5.3.2.5 Open template and save with name of procedure and new calibrator lot number

5.3.2.6 Right click on the worksheet tab, select “move or copy”, check “create a copy” and select “move to end”. Repeat for every analyte included in the calibration samples.

5.3.2.7 Rename each sheet to match analyte being evaluated

5.3.3 **Acceptance Criteria**

5.3.3.1 When the current calibrator lot results and new calibrator lot results are entered to the spreadsheet, a chart will be generated comparing the two sets of data.

5.3.3.2 The slope of best-fit line is acceptable if:

5.3.3.2.1 It is between 0.85 and 1.15, and either

5.3.3.2.2 It is between 0.95 and 1.05, or

5.3.3.2.3 The uncertainty range (95% confidence interval) contains 1.

5.3.3.3 The y-intercept of the best fit line is acceptable if:

5.3.3.3.1 The uncertainty range (95% confidence interval) contains 0.

5.3.3.4 The QC values when calculated vs. the new calibrator must be within the % acceptance used when validating the particular QC

5.3.3.5 If criteria are met, the pass/review fields will read, “Pass”.

5.3.3.6 If the criteria are not met, the pass/review fields will read “Review”.

5.3.3.7 Further supplemental information is available to assist in evaluating how the calibrators compare:

5.3.3.7.1 The % difference between the two calibrator results will be calculated, and will be shaded if the new calibrator is more than ±15% different than the current calibrator.

5.3.3.7.2 The bias from the new calibrator to current calibrator is calculated.

Note: The calculations used in the excel spreadsheet are described in the "calculations" Tab.

5.3.4 **Review/approval process:**

5.3.4.1 After all appropriate data has been entered to the file, it is to be saved and the appropriate Supervisor, Team Leader, or designee is to be notified.

5.3.4.2 If all criteria are met, the supervisor, team leader, or designee can enter "yes" in the approval field and enter their name and date in the review area, thereby approving the new calibration lot.

5.3.4.3 If any criteria are not met, review of the new lot of calibrator can only be performed by the supervisor.

5.3.4.4 If upon further review, the supervisor decides that the new calibration lot is acceptable, appropriate comments are to be placed in the Comments field specifying why it was accepted. The supervisor must then enter "yes" in the approval field, along with their name and date in the review area, thereby approving the calibration lot.

5.3.4.5 If upon further review, the supervisor decides that the new calibration lot is not acceptable, appropriate comments are to be placed in the Comments field specifying the appropriate steps to be taken. The supervisor must then enter "no" in the approval field, along with their name and date in the review area, thereby verifying that the review is complete.

## 6 VALIDATING AND RE-VALIDATING INTERNAL STANDARDS

### 6.1 Validating a New Lot of Internal Standard

6.1.1 Add the amount of internal standard noted in the bench procedure to blank matrix. Run in duplicate.

6.1.2 Compare the area(s) or height(s) of the new internal standard to the area or height of the current internal standard in a blank matrix sample.

6.1.3 The two results should match within +/- 30%. For results outside this range, consult a supervisor or manager.

### 6.2 Re-validating an Existing Lot of Internal Standard

6.2.1 Prior to the expiration date, the current lot of internal standard should be evaluated to determine if the expiration date can be extended. Compare the internal standard in a current run to the chromatograms from the original validation.

6.2.2 Fortify a blank matrix sample with the normal amount of IS

6.2.3 Evaluate the blank matrix to ensure the internal standard has not degraded or become contaminated

6.2.4 Any peak for the analyte of interest should be < 10% of the area or height observed in the lowest calibration sample.

6.2.5 Using at least two blank matrixes from the current run, compare the area or height of the internal standard to the original validation data - The internal standard should be within +/- 30% of the validation data.

6.2.6 If the internal standard meets acceptance criteria place a new label on the internal standard bottle, and attach re-validation paperwork to original paperwork.

6.2.7 If the internal standard is deemed unacceptable after investigation prepare a new lot of IS

and validate as described above.

## 7 ESTABLISHING TARGET CONCENTRATION AND ACCEPTANCE RANGE FOR QC SAMPLES IN QUANTITATIVE ASSAYS

### 7.1 Commercial controls

Follow the manufacturer's instructions for material preparation, and then perform 4 separate analytical runs with 3 replicates per run or a minimum of 12 replicates over more than one run to establish the mean. Perform these determinations in parallel with the existing controls to verify performance. Acceptable criterion is that the mean value is within ±15% of nominal. The controls should have verified QC result data from the manufacturer whenever possible to designate the nominal value. This validation is conducted if the LOT number for commercial controls changes.

### 7.2 In-House controls

The target is defined to be the average calculated concentration of a total of at least 12 analyses performed in a minimum of 4 separate analytical runs, with a minimum of 3 replicates per run. The verified target must be within ±15% of nominal concentration.

7.3 Daily spiked QCs: The target is defined as the nominal value

## 8 ESTABLISHING PERFORMANCE FOR QC SAMPLES FOR QUALITATIVE ASSAYS

### 8.1 Commercial controls

Follow the manufacturer's instructions and perform a minimum of 4 separate analytical runs with a minimum of 3 replicates per run. Perform these determinations in parallel with the existing controls to verify performance. All samples must provide accepted results.

### 8.2 In-House controls

8.3 The target is defined as the average calculated concentration of a total of at least 12 analyses performed in a minimum of 4 separate analytical runs, with a minimum of 3 replicates per run. All samples must provide expected results

## 9 VALIDATION OF NEWLY PREPARED REAGENTS

9.1 Run a set of controls using the newly prepared reagent in parallel with controls made using the existing reagent.

9.2 The reagent will be considered validated if controls meet the acceptance criteria defined by the method

# In-Process Calibration and Quality Control for Drug Screening/Confirmation Testing

## 1 PURPOSE

This procedure describes the preparation and implementation of a calibration curve and in-process quality control (QC) samples at Houston Forensic Science Center. This procedure is designed to provide a means of detecting potential problems with assay performance and to ensure accurate and reliable test results.

## 2 SCOPE

These are default procedures for QC of all validated qualitative and quantitative bio-analytical assays applicable to all analytical SOPs not having specific QC protocols.

## 3 DEFINITIONS

3.1 Calibration Protocol: A written procedure, which describes the preparation of calibration samples, the processing of these samples and the method-specific calculation model that is to be used.

3.2 Calibration sample: An analytical standard used to fix, set or check the graduations or scale of an analytical procedure.

3.3 Carryover: An analyte that is retained from one sample into another sample, usually the sample immediately following one that has an elevated concentration of the analyte of interest.

3.4 Certified Reference Material (CRM): drug standard purchased from an approved vendor which includes a certificate of analysis verifying the concentration.

3.5 Fortified Quality Control Sample: A sample of similar matrix to the unknown case sample, which has been spiked with a predetermined amount of the analyte(s) of interest.

3.6 Internal Standard: An analyte (generally of similar chemical structure to an analyte being measured) that is added, in a known concentration, to all samples (calibrators, QC's, unknowns) in an analytical method, and that functions as a reference marker for that sample, against which the analyte of interest can be measured.

3.7 Linear Range: Typically the Limit of Quantification (LOQ) to the Upper Limit of Quantification (ULOQ) are administratively defined as the concentration of the lowest and highest calibrator used in preparation of the calibration curve.

3.8 Matrix: The material into which is spiked known amounts of an analyte(s) of interest in order to calibrate the method or to track method performance.

3.9 Neat: A systematic representative of an analyte of interest that is free from admixture or dilution.

3.10 Negative control: matrix fortified with Internal Standard. The negative control may also contain the analyte of interest at a concentration below the Limit of Quantification or Cutoff of the assay.

## 4 CALIBRATION OF QUANTITATIVE ASSAYS

4.1 Calibration protocol must be performed as validated and described in the analytical method.

4.2 A matrix blank must be included in each analytical run but this blank will NOT be used in the generation of the calibration curve unless specifically indicated in the analytical method.

4.3 Unless otherwise specified in the analytical procedure, no fewer than three calibration levels, spanning the linear range of the assay (not including the blank), may be used for a linear

calibration curve. The three concentrations must span the linear range of the assay.

4.4 No fewer than 4 non-zero points spanning the analytical range of the assay may be used for a quadratic curve at any time.

4.5 For the calibration curve to be accepted, the back-calculated results for each calibrator must calculate to within ± 20% of its target value. A variance of ± 25% is allowed at the LOQ.

4.6 In some situations one point may be eliminated from the calibration curve to improve the quality of the curve fit. This does not include the blank. Elimination of two points should only be made in exceptional circumstances when other evidence supports the use of analytical data from that particular analysis batch. The approval of a supervisor or designee is required when two points are discarded.

4.7 When a calibrator is discarded, the fact that it was discarded and the reason must be clearly documented with the data for that batch.

4.8 The lowest acceptable calibrator for a given batch is the reporting limit, unless otherwise specified in the analytical method.

4.9 A negative control should be included after the highest calibrator in each analytical run to monitor for carryover.

## 5 CALIBRATION OF QUALITATIVE ASSAYS

5.1 A cutoff calibrator must be included in every analytical run

5.2 Results of unknown samples are determined to be positive or negative when evaluated against the response of the cut-off calibrator.

## 6 CONTROL OF QUANTITATIVE ASSAYS

6.1 Each analytical run must contain a matrix blank, two low QC sample and two high QC sample

6.1.1 Low QC concentration should be no more than 3x the LOQ of the assay

6.1.2 High QC concentration should be no less than 75% of the highest calibrator

6.2 The number of QC samples must be at least 5% of the total number of unknown samples in the run.

6.3 A low QC sample should be injected after the last case specimen for each run.

6.4 QC samples must be included for every analyte being quantified by the method.

6.5 Acceptance criteria for QC samples should be defined in SOP of each analytical assay.

## 7 CONTROL OF QUALITATIVE ASSAYS

7.1 Each analytical batch of a qualitative assay must include a matrix blank and at least one positive control, i.e. a sample of the control matrix fortified to a concentration within 3 times that of the reporting limit for the assay with all drug classes or individual compounds for which the assay is designed to detect. For immunoassay methods the positive control may be spiked at a concentration 200% above the positivity threshold.

7.2 Each analytical batch must include a low QC sample fortified at no less than 50% of the cutoff calibrator concentration.

7.3 A run may be accepted if system suitability (if applicable) is acceptable, if all QC samples perform as expected, i.e., negative controls and reagent blank give a negative response and positive control(s) gives a positive response.

# General Guidelines for Instruments and Equipment

1 **PURPOSE**

Instrumentation and equipment must be regularly maintained to ensure precision and accuracy in the various assays used by the toxicology section.

2 **SCOPE**

These guidelines are intended to describe proper operation, maintenance, and performance verification procedures for key instrumentation.

3 **MASS SPECTROMETERS**

3.1 Tune Verification and Autotunes

3.1.1 A tune verification must be performed each day the instrument is used to evaluate the instrument's performance and to check for leaks. An autotune must be performed after any maintenance and may be done at other intervals as deemed necessary by the analyst. During an autotune, the MSD is calibrated by tuning the instrument to ensure that the mass-to-charge ratios (m/z) are assigned correctly and that the scan ratio is set properly. This procedure also serves as a check for air leaks.

3.1.1.1 Each day that a tune verification or an autotune is performed, it should be documented on the GC/MS Maintenance log form LAB-24 or an equivalent form. A copy of the most recent tune file should be recorded in a retrievable format.

3.1.1.2 Following an EI autotune or tune verification on the mass spectrometer, the tune report should be examined. If the tune does not meet the criteria for the application, then action should be taken to determine why it does not meet said criteria. For example, the system may need a refreshed autotune, a source cleaning or there may be an air leak.

3.1.1.3 If an instrument does not pass the tune verification, no casework will be performed using that instrument until the problem is resolved and the tune verification falls within acceptable specifications.

3.1.1.3.1 Tune Specifications

3.1.1.3.1.1 The three tuning masses must be within ±0.2 amu of 69.00, 219.00, and 502.00.

3.1.1.3.1.2 The peak widths of the three tuning masses must be within ±0.10 amu of 0.60 amu.

3.1.1.3.1.3 The ratio of mass 70 to 69 must be from 0.5 – 1.6%.

3.1.1.3.1.4 The ratio of mass 220 to 219 must be from 3.2 – 5.4 %

3.1.1.3.1.5 The ratio of mass 503 to 502 must be from 7.9 - 12.3%

3.1.1.3.1.6 The ratio of mass 219 to 69 must be >40%

3.1.1.3.1.7 The ratio of mass 502 to 69 must be > 2.4%

3.1.1.3.1.8 The precursor for mass 69 must be ≤3%

3.1.1.3.1.9 The precursor for mass 219 must be ≤6%

3.1.1.3.1.10 The abundance of any peaks less than 69 amu must not be greater than 10% of the base peak abundance. Peaks at 18, 28, and 32 amu are indicative of water, nitrogen, and oxygen, respectively, and may indicate an air leak.

3.1.2 Maintenance

3.1.2.1 Maintenance should be performed following the manufacturer's guidelines or more frequently as needed. Refer to Agilent 5975 Series MSD Operation Manual or equivalent. All maintenance and repairs should be documented on the GC/MS Maintenance Log Form LAB-24 or an equivalent form.

3.1.2.1.1 Before each use:

3.1.2.1.1.1 Perform a tune verification

3.1.2.1.1.2 Verify tank pressure

3.1.2.1.1.2.1 Check the wash solvents (note: The solvent vials may be rinsed and filled or refilled as needed.)

3.1.2.1.1.3 Weekly – Check the foreline pump oil level

3.1.2.1.1.4 Monthly – Check the o-ring and change as needed

3.1.2.1.1.5 Every six months to one year

3.1.2.1.1.5.1 Replace the foreline pump oil

3.1.2.1.1.5.2 Check calibration vial and refill PFTBA as necessary

3.1.2.1.1.5.3 Check diffusion pump oil and replace if necessary

3.1.2.1.1.6 As needed (depending upon instrument and sample throughput)

3.1.2.1.1.6.1 Change the septum

3.1.2.1.1.6.2 Check and replace the inlet liner

3.1.2.1.1.6.3 Check and replace the gold seal

3.1.2.1.1.6.4 Clip the column

3.1.2.1.1.6.5 Replace/Switch filament(s)

3.1.2.1.1.6.6 Replace gas cylinders

3.1.2.1.1.6.7 Clean the ion source

3.1.3 Methods

Electronic backups of the methods and data files are recommended for each instrument. An electronic copy of the method is located in the Instrument Method Folder or equivalent. Methods are updated regularly following routine instrument maintenance. It is recommended that the examiner report, initial, and date any updated methods.

3.1.4 Sample Preparation and Sequence Set-up

3.1.4.1 Samples should be prepared for analysis according to the section's standards or specific SOP.

3.1.4.2 The data file path must clearly identify the location and storage of the data. The convention for the data file storage should include the date and name of the examiner.

3.1.4.3 Retain GC/MS analysis data in the case record.

## 4 PIPETTES

4.1 Method of Use

Refer to appropriate manuals for proper handling and use.

4.2 Performance Verification and Maintenance

4.2.1 Each pipette should be externally calibrated and certified by an approved calibration vendor once per calendar year.

4.2.2 Pipettes that dispense volumes greater than 25 µL are evaluated twice per calendar year using a combination of external calibration by an approved vendor and internal performance verification procedures.

4.2.3 Due to the inability to gravimetrically verify the small volumes (2.0 µL) associated with low volume pipettes, these are not subject to in-house verification. These pipettes are calibrated and certified annually by an outside vendor.

4.2.4 Pipette verifications are also performed following any routine maintenance or cleaning.

4.2.5 To check for accuracy, room temperature deionized water should be pipetted into a weighing vessel on an analytical balance.

4.2.6 The pipette should be checked using 3-points over the full range with 5 replicates at each volume.

4.2.7 The weight of the water delivered should be recorded on the form LAB-41 Pipette Performance Verification Check or by an equivalent method.

4.2.8 The average value of each pipette (Air and Positive Displacement) should fall within the verification tolerance ranges set by the external vendor's calibration document (±3%) before it may be used for casework.

4.2.9 Follow manufacturer's instructions for troubleshooting maintenance if needed.

4.2.10 If a pipette fails a performance verification check or if an analyst has reason to believe that a pipette is not working properly they must:

4.2.10.1 Perform a pipette verification and if the pipette is not in proper working order:

4.2.10.1.1 Clearly mark the pipette "OUT OF SERVICE".

4.2.10.1.2 Inform the laboratory manager. No laboratory case work will be performed using the pipette until the problem is corrected.

4.2.10.1.3 Repair or send out the pipette for repairs.

4.2.10.1.4 Verify that the pipette is working correctly and falls with-in the proper tolerances following routine repair, maintenance, or calibration.

4.2.10.1.5 Maintain the appropriate documentation and update the log book.

4.2.10.2 Occasionally, a pipette may be out of service, even if no problem has been identified – newly purchased pipettes pending verification or calibration. In the event a pipette is out of service (inactive, in repair, etc.) the physical pipette shall be marked "OUT OF SERVICE" and the appropriate dates for the period documented (LAB-41, Pipette Performance Verification Check or an equivalent form).

4.2.10.3 A volumetric or positive displacement pipette is intended for the quantitative transfer of a liquid. On occasion, however, pipettes are used only for qualitative purposes (e.g., transfer steps during derivatizations). All pipettes are subject to external calibration and internal performance verification unless they are identified as "OUT OF SERVICE" or "QUALITATIVE ONLY".

5 **PH METER**

Three-Point Calibration

5.1 Refer to the User's Manual for detailed instructions on proper handling and use.

5.2 A three-point performance check is a more accurate and preferred method for measuring pH of a solution when pH accuracy better than ± 0.1 is required. It is recommended that this check is performed monthly or as needed depending on use.

5.3 At time of use, if a three-point check has not been done, the pH meter should be verified using a pH buffer as close to the sample pH as possible.

## 6 REFRIGERATORS

6.1 Maintenance and Performance Verification

6.1.1.1 Refrigerators and freezers should remain clean and organized at all times. If a spill occurs, appropriate cleaning procedures should be performed.

6.1.1.2 Evidence must be kept separately from drug standards, reagents, and other analytical substances by storage in a separate refrigerator or freezer.

6.1.1.3 Toxicology evidence is routinely stored in refrigerators within the toxicology section. Freezer storage is an acceptable alternative. All refrigerators and freezers in the toxicology section are monitored using TempAlert, which is further detailed in the Quality Manual, or equivalent system.

Acceptable refrigerator temperature range: > 0 - 10°C

7 Acceptable freezer temperature range: ≤ 0°C

7.1.1.1 If a refrigerator or freezer is open for an extended period (e.g., cleaning, inventory), this should be documented on form LAB-47 (Universal Maintenance, Repair and/or External Calibration log) or by an equivalent method.

7.1.1.2 If a refrigerator/freezer stops functioning and exceeds the acceptable temperature range, evidence will be moved to another functioning refrigerator/freezer and transfer documented.

7.1.2 NIST Traceable Thermometers

7.1.2.1 Temperature measurements for refrigerators, freezers, and chart recorders should be performed using NIST traceable thermometers.

7.1.2.2 If any thermometer fails performance verification or is suspected of not working properly, laboratory management should be notified and a record made in the instrument lab book or equivalent. The manual may be used for troubleshooting, but if the problem is not resolved in a timely manner, it should immediately be removed from service. Once repaired and verified to be within acceptable specifications, the equipment may be returned to service.

## 8 BALANCES

8.1 Method of Use

8.1.1 Refer to the appropriate operating instructions for proper handling and use.

8.2 Performance Verification and Maintenance

8.2.1 Performance verification and maintenance should be performed following the manufacturer's guidelines and the schedule described below.

8.2.1.1 Balances

8.2.1.1.1 Balances must be calibrated and certified with a traceable certificate by an external vendor once a year.

8.2.1.1.2 An internal performance check must be conducted prior to each use using NIST-traceable reference weights. If an internal performance check on a balance has been completed on a particular day, the balance does not need to be checked again for the day. This is documented using form LAB-12 Balance and Weights Performance Check or an equivalent method.

8.2.1.1.3 Additional performance verifications may be performed as necessary.

8.2.1.1.4 Balances should be checked for accuracy each time the balance is moved and after maintenance is performed.

8.2.1.2 Weights

8.2.1.2.1 Weights used to performance check the balance shall be sent to a vendor for re-certification every 3 years.

8.2.1.2.2 Laboratory weights should be inspected after the annual recertification of the balance.

8.2.1.2.3 Laboratory weights should be stored, transported, and handled using precautions to protect them from contamination and deterioration.

8.2.1.2.4 It is recommended that internal performance verification be performed at least annually. This is documented using form LAB-12 Balance and Weights Performance Check or an equivalent method.

8.2.1.3 General

8.2.1.3.1 Maintain a log book, or electronic equivalent, with the results of performance verifications of balances and weight sets, maintenance, and certification.

8.2.1.3.2 If the result from a performance check is outside of the acceptable range, the balance will be immediately taken out of service until maintenance and/or certification are performed by an approved vendor. Laboratory management should be notified and the problem is to be documented in the instrument log book, or an electronic equivalent.

8.2.1.3.3 Since the tolerances of electronic balances vary, instrument specifications must be checked to determine the appropriate criteria for satisfactory performance. The following general specifications may be used.

| Balance Class | Weights | Acceptable Range |
|---|---|---|
| Analytical | 0.002 g | 0.0015-0.0025 g |
| | 1 g | 0.95-1.05 g |
| | 5 g | 4.95-5.05 g |
| Top Loading | 1 g | 0.95-1.05 g |
| | 5 g | 4.95-5.05 g |
| | 100 g | 99.5-100.5 g |

## 9 HEATING BLOCK

9.1 Method of Use

9.1.1 Refer to the appropriate operating instructions for proper handling and use.

9.2 Performance Verification and Maintenance

Temperature of heating blocks should be measured with a thermometer prior to each use. The value should be within ±5°C of the optimal temperature for relevant analytical assays. This is documented on Form LAB-69 Temperature Log or equivalent method.

## 10 LITERATURE REFERENCES

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10.3 Agilent Technologies. Agilent 5975 Series MSD: Operation Manual, Part Number G3170-90030, 2010.

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10.5 Agilent Technologies. Agilent MSD ChemStation Software: User Information CD, Part Number G1701-60125.

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10.18 VWR. VWR Chromatography Refrigerator Manual, 2007.

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# Cleaning and Deactivating GC/MS Injection Liners (Silanizing Glassware)

## 1 PURPOSE

The use of silanized glassware may be necessary for some toxicological procedures. A silanizing reagent is used to deactivate the surface of the glass and reduce adsorptive losses that may occur with polar drugs (and/or metabolites), particularly those that may be present at very low concentrations. Refer to the respective procedures to determine if silanized glassware is necessary.

## 2 SCOPE

The continued use of a GC-MS liner will eventually result in the accumulation of contaminants in the liner, causing a loss in performance, and making it unsuitable for use. Following routine use, GC-MS liners can be reused if appropriate cleaning and chemical deactivation steps are followed. This SOP describes the cleaning, deactivation, and silanization process.

## 3 REAGENTS

3.1 Chromic Acid

3.1.1 May be prepared using concentrated sulfuric acid and potassium dichromate.

3.1.2 A recommended preparation includes 20 mL potassium dichromate, 60 mL concentrated sulfuric acid and 200 mL deionized water. Quantity may be increased as needed using appropriate ratios (1:3:10).

3.1.3 The solution should be a dark orange-red color; should it turn green, it should be disposed of using proper disposal procedures.

3.2 Silanizing reagent: 1,1,1,3,3,3-Hexamethyldisilazane.

**CAUTION:** Flammable liquid, extremely harmful when inhaled and ingested, extremely corrosive to skin, mucous membranes, and respiratory tract. May cause eye burns.

## 4 EQUIPMENT AND MATERIALS

4.1 Hot Plate

4.2 Beakers

4.3 Forceps

4.4 Pasteur pipettes

4.5 Sonicator

4.6 Vacuum oven

4.7 Hamilton 500 µL syringe or equivalent

## 5 PROCEDURE FOR CLEANING AND DEACTIVATION

5.1 Carefully place the chromic acid solution on a hot plate in a hood and heat to approximately 80 °C.

5.2 Using forceps, gently place the liners in the chromic acid solution with the top of liner pointing up.

5.3 Using a Pasteur pipette, continuously fill the liners with the chromic acid solution. Allow the solution to move through the liners cleaning and removing interior soiling.

5.4 When all soiling appears to be removed from the interior, carefully transfer the liners to a clean beaker to finish draining. Heavily soiled liners may need more rinses in the chromic acid solution to become clean.

5.5 Carefully rinse the beaker with the drained liners with deionized water in order to remove all traces of the chromic acid solution. Individual liners may be rinsed with deionized water to facilitate this process.

5.6 Sonicate the liners in deionized water for 10 minutes. Repeat with fresh deionized water.

5.7 Sonicate the liners in methanol for 10 minutes. Briefly allow the liners to dry.

5.8 Cleaned and deactivated liners should be handled only with gloves and should be stored in a location to protect them from possible sources of contamination.

5.9 Liners will be silanized prior to use.

## 6 PROCEDURE FOR SILANIZATION

6.1 Place glassware items to be silanized into the vacuum oven. Ensure that glassware does not obstruct the flow of gases or vapors.

6.2 Heat the oven to 160 °C.

6.3 Ensure that both vent valves are closed.

6.4 Turn on the vacuum and slowly open the vacuum valve to allow the vacuum to rise. The needle on the gauge will move.

6.5 When the vacuum reaches ~ 15 psi, close the vacuum valve.

6.6 Turn off the vacuum.

6.7 Prepare the injection by drawing up 100 µL of 1,1,1,3,3,3-Hexamethyldisilazane into a Hamilton gas tight syringe. Carefully open the injector valve just until the vacuum begins to steadily fall. Inject the 1,1,1,3,3,3-Hexamethyldisilazane. The oven must keep a vacuum of at least -10 psi during silanization.

6.8 Close the injector valve.

6.9 Silanize glassware for at least 1 hour.

6.10 Open the vacuum valve so that the vacuum starts to fall gradually to zero.

6.11 Vent the oven by opening the injection valve.

6.12 Turn off the oven and allow the contents to cool to room temperature.

6.13 Remove the glassware. Store silanized glassware in a clearly marked receptacle.

## 7 LITERATURE AND SUPPORTING DOCUMENTATION

7.1 Operator Manual, NAPCO Vacuum Oven, Thermo Scientific; Manual P/N 3177871.

# Operation and Maintenance of Zymark Turbo Vap LV

1 **PURPOSE**

During toxicological extraction, samples require evaporation in order to concentrate the isolated material. The Zymark Turbo Vap® LV is a microprocessor-controlled evaporator that provides simultaneous and automated concentration of multiple samples, unattended operation, convenience, and speed. The Turbo Vap will allow high capacity evaporation at a maximum of 50 samples processed simultaneously.

2 **SCOPE**

The Turbo Vap will be used routinely during extractions in order to concentrate extracts or as needed. Analysts should be knowledgeable regarding the use, precautions, and maintenance of this evaporator.

3 **EQUIPMENT**

3.1 Zymark Tubo Vap LV Workstation and accessories
3.2 Compatible test tube rack
3.3 Inert gas supply (nitrogen)
3.4 Distilled or deionized water
3.5 Timer
3.6 Siphon tube
3.7 Spectrum Clear Bath

4 **PRECAUTIONS**

4.1 The workstation must be placed in an appropriate location with available gas and electrical sources, as well as adequate ventilation. The workstation may either be placed inside a fume hood or the exhaust duct supplied with the unit must be utilized. This *MUST* go to a suitable ventilation system vented *outside* the laboratory.

**Note:** Exhaust gases may be hazardous. Consult the Material Safety Data Sheets for all solvents used.

4.2 The workstation must be used on a flat, level, stable surface.
4.3 The workstation must never be used with hydrogen or other flammable gases which may explode or catch on fire.
4.4 DO NOT move the unit when the bath is full of water. It is a burn hazard.
4.5 DO NOT operate the Turbo Vap LV without water in the bath to avoid the risk of fire or burn injuries.
4.6 To avoid injury, DO NOT EXCEED 100 psi maximum inlet pressure.

5 **PROCEDURE**

5.1 Turn on the unit and gas supply.

5.2 Check the water bath and fill to the appropriate level with distilled or deionized water. The water bath level should be AS HIGH AS the initial level of solvent in your sample tubes unless you are using micro-centrifuge racks and tubes.

5.3 Check the gas supply and pressure

5.4 Set the water bath temperature as specified in the respective procedure. The bath will be at the correct temperature when the TEMP light stops blinking, in approximately 20 minutes.

5.5 Set the gas pressure and time setting in accordance with the SOP.

5.6 Turn on the rows containing sample tubes by pressing the corresponding tube stations pushbuttons. There are 5 rows of 10 stations available for use. Partial rows may have unused gas nozzles. These should be plugged with supplied stoppers for optimum gas flow and sample protection against moisture.

**Note:** Leaving empty rows unplugged while in use can cause condensation on the unit lid, which may in turn contaminate the sample.

5.7 Load sample tubes into the evaporator by opening the cover and placing the sample racks into the water bath.

5.8 Close the cover and press START. To stop an evaporation run, simply press STOP. To pause an evaporation run, press the START/PAUSE pushbutton. To shut off the evaporation in any row of test tubes, press the corresponding TUBE STATIONS pushbutton.

5.9 When the cycle is complete, the gas automatically shuts off and the evaporator buzzer will sound every 30 seconds. Lift lid and leave open as soon as possible. Remove tubes and blot with absorbent material to remove moisture if desired.

**Note**: highly volatile samples can be lost if they are allowed to remain in the unit.

5.10 When use of the TurboVap is complete for the day, turn the unit power and gas supply off. Lift the cover and keep it open.

## 6 MAINTENANCE

6.1 Routine maintenance of the Turbo Vap LV eliminates the need for frequent cleaning due to cloudy or bacteria infested water.

**Note:** Cleaning the water bath may cause exposure to bacterial or viral hazards. Use good laboratory operating procedures when dealing with liquids.

6.2 Turn the evaporator's AC power OFF and unplug the power cord.

6.3 Open the cover and remove the rack.

6.4 Siphon the water out of the bath.

6.5 Use an appropriate cleaner if desired, to wipe any residue from the bath walls. Rinse the bath and re-siphon the liquid. Also, clean the rack itself.

6.6 Pour ~1 liter distilled or deionized water into bath. Add 15 drops of *Clear Bath*.

6.7 Add more water until liquid level is at standard operating height.

6.8 Refer to user manual for other maintenance issues including but not limited to fuse replacement, leak checks, and troubleshooting.

## 7 LITERATURE AND SUPPORTING DOCUMENTATION

Operator's Manual. TurboVap LV Evaporator Workstation. Zymark Corporation. P/N 71467. Rev. 3.

# Operation and Maintenance of CEREX Pressure Processors

## 1 PURPOSE

During solid phase extraction (SPE), samples require processing and drying which may be carried out using positive pressure. The CEREX® Pressure Processor is equipped with 48 individual, restricted gas ports to allow for positive pressure processing of solid phase extraction columns. It is also equipped with on/off switches, in order to preserve gas and save time by not having to plug ports not in use.

## 2 SCOPE

Each of two positive pressure manifolds, CEREX System 48 and CEREX System 48-II, will be used routinely during solid phase extraction in the toxicology section of the laboratory. Analysts should be knowledgeable regarding use and maintenance of these processors.

## 3 EQUIPMENT

3.1 CEREX System 48 ("System 48") or CEREX System 48-II ("CEREX 48")
3.2 Collection tube rack, 16x100mm
3.3 SPE Rack, 6CC, for 48 Place Processor
3.4 Waste bin(s) and waste bin rack
3.5 Nitrogen and Air compressed gas cylinder/supply

## 4 PRECAUTIONS

4.1 The SPE Processor does not include a filter on the source gas input. A clean, oil-free gas source is essential to prevent sample contamination.
4.2 Compressed nitrogen is the recommended pressure source. If necessary, the unit can operate using high purity filtered air.

> **Note:** The positive pressure processor must be used in a fume hood in order to prevent inhalation of aspirated biological fluids and organic solvents.

## 5 PROCEDURE

5.1 Once samples are ready for extraction, place SPE columns into SPE rack.
5.2 Place a waste bin in the waste bin rack and set the SPE rack on top. The black marker on the rack leg should be in the back right corner for correct alignment of racks, column openings, and gas flow.
5.3 Turn on gas flow to the unit.
5.4 To compress the manifold, place the assembled collection and column racks on the slide platform. Slide rack to the back of the unit using the small handle on the front of the slide platform. The platform will stop when it reaches the stop located at the back of the unit, under the manifold.
5.5 To begin applying pressure to the rack assembly:
5.5.1 System 48: Simultaneously lift the black switches on each side of the unit. This will move the rack assembly up and against the manifold.

5.5.2 CEREX 48: Simultaneously push the blue buttons on each side of the front of the unit until the manifold moves down onto the rack assembly.

**Note:** Releasing the blue buttons too soon will cause the depression mechanism to automatically open and return to the manifold to its original position.

5.6 Using the 4 toggle switches on the top of the manifold, turn on the rows that will be needed. (If rows are not needed, they may be turned off, if desired, to save nitrogen.)

5.7 To adjust the flow, use the "SPE flow rate" knob located on the front of the unit. Three different settings are available to use during extraction.

5.7.1 'Off' – No gas flows to the manifold. This setting should be used when compression and decompression of the manifold are taking place.

5.7.2 'Adjust Flow' – The gas is delivered through the ADJ FLOW regulator and then through the rotameter which is controlled by the needle valve located at its base. This flow is optimal when precise, slow flow is required for the columns. This is the most gentle, limiting flow setting.

5.7.3 'Max Flow' – The gas delivery system provides rapid gas flow to the manifold which can be controlled by adjusting the regulator located below the 'Max Flow' pressure gauge on the front of the unit. This pressure range can be used to maximize flow through the columns and during column drying.

**Note:** The 'max flow' regulator is a 'locking' knob. It must be adjusted by pulling out the knob. Once the desired pressure is set, it can be 'locked in' by pushing in on the knob.

5.8 Decompression of the manifold is done by the following procedure

5.8.1 System 48: Simultaneously push down the black switches on each side of the unit. This will move the rack assembly down and away from the column assembly.

5.8.2 CEREX 48: Simultaneously push the blue buttons on each side of the front of the unit until the manifold moves up, away from the rack assembly.

## 6 MAINTENANCE

Both System 48 and CEREX 48 are constructed of anodized aluminum, stainless steel, and solvent resistant plastics. However, the following items should be observed during the operation:

6.1 Solvent spillage or overflow should be cleaned immediately in order to prevent instrument damage.

6.2 The column seal is silicone rubber and should be cleaned with methanol after each use.

## 7 LITERATURE

Operating Instruction. System 48-II CEREX Pressure Processor. CEREX 48-II manual rev. B.

# Operation and Maintenance of the TECAN Freedom Evo 75

1 **PURPOSE**

The TECAN Freedom Evo 75 is an instrument programmed to aliquot fixed quantities of sample and reagent into 96-well microliter plates for use in the Enzyme-Linked Immunosorbent Assay (ELISA).

2 **SCOPE**

This document describes the operation and maintenance of the Tecan Freedom Evo 75.

3 **EQUIPMENT**

TECAN Freedom Evo, Model: FREEDOM Evo 75

4 **REAGENTS AND MATERIALS**

4.1 Commercial immunoassay kits ("strips") for drugs-of-abuse testing.

4.2 Deionized water.

4.3 1M HCl (hydrochloric acid): Add 84.6 mL concentrated hydrochloric acid (HCl) to a 1 liter volumetric flask containing deionized water. Bring to volume with deionized water. Store at room temperature (12 month expiration).

4.4 1 M NaOH (sodium hydroxide): Dissolve 40.0 g sodium hydroxide pellets in 1L deionized water. Store at room temperature (12 month expiration).

5 **INSTRUMENT OPERATION:**

5.1.1 Place the subsample tubes into racks in accordance with the plate map. Place a high QC after every 10 samples, and after the last sample in the run. Make sure to place the conjugate tubes with the conjugate solution corresponding to its microplate. All reagents, samples and strips must be at room temperature before the start of the run.

5.2 **Flushing Tips**:

5.2.1 Double click on "Evoware Standard" icon.

5.2.2 Enter username and password and click enter.

5.2.3 Check "Edit and existing script".

5.2.4 Click the "Start your Selection" arrow.

5.2.5 Click on favorites; double click on "PP_2TipN02_96".

5.2.6 Click on the "Commands" tab. Double click on "Flush".

5.2.7 Make sure path is clear for moving the tips.

5.2.8 Click "Start Flushing Tips". The instrument flushes both tips with deionized water before starting the run.

5.2.9 Click "Ok".

5.2.10 Repeat flushing for at least 4 cycles (repeat steps 7 & 8) to prime the instrument. Check to see if any bubbles are trapped in the instrument's tubing.

5.2.11 When flushing is complete, go to "File", click "Exit, unload drivers".

5.2.12 The display screen stating “Should Evoware move all arms to their home positions?” Click Yes.

5.3 **To Start Running a Sample Set Using NaviTrak-Operating System (OS):**

5.3.1 Double click “NaviTrak2” icon and click “start”

5.3.2 Under the “Test Panel” tab, click “Recall Panel”

5.3.3 For analyzing blood samples and all assays click “blood.pnl.” This automatically brings up the panel that has the aliquots of blood that need to be added into the wells.

5.3.4 Similarly, for urine samples and all assays click “urine.pnl.”

5.3.5 Verify the “TMB on the deck” is checked.

5.3.6 Under the “Sample List” tab, create the sequence by filling out the “ID” column of the second box (controls, samples, and a high QC after every 10 samples and the end of the sequence).

5.3.7 Enter sample IDs reflecting the order of the samples in the sample tray, no two boxes can have the identical name (i.e. High, High2).

5.3.8 Click “Save Sample List” print sequence by clicking “Print Samples List”, and save run by clicking “Save Job.”

5.3.9 Take a printout of the sample list and place in batch folder.

5.3.10 Review the sequence and sample IDs under the “Review” tab.

5.3.11 Click the “Run Evo” button to start run. The dialogue box displays if the machine detects any error. The instrument pipettes the indicated amount of controls and samples into the wells in each plate and 100 µL of the conjugate solution. A timer automatically pops up. Allow the timer to proceed through the incubation countdown.

5.3.12 Incubate for 1 hour, away from light.

5.3.13 After incubation, a command box appears instructing to wash the plates in the plate washer and replace them on the deck. (See procedures for operation of plate reader.)

5.3.14 After washing the plates, place them back into their respective places and pour the TMB substrate into the TMB substrate container. Place the TMB substrate container into its respective position on the instrument. Click “OK”.

5.3.15 The instrument pipettes 100 µL of TMB substrate into the washed wells. Incubate for 30 minutes, away from light.

5.3.16 Place the plates back into their respective places on the instrument prior to the completion of the 30 minute incubation period.

5.3.17 After 30 minutes of incubation, the instrument automatically pipettes 100 µL of STOP solution into the wells. The plates are ready to be read on the plate reader.

5.4 **For analyzing individual assays**

5.4.1 To enter the aliquots manually for blood and urine runs, select “Unlock.”

5.4.2 Right click the panel to add or delete a microplate

5.4.3 Microplate names can be changed by clicking in the associated white box. Verify that the amount to be aliquoted is correct.

5.4.4 Click on the white drop down box and select the ELISA kit test numbers based on the drug that needs to be tested on the microplates.

5.4.5 Check the “TMB on the deck” box.

5.4.6 Click the "Lock" button when complete and saving the panel is optional, by clicking "Save Panel."

## 6 MAINTENANCE OF THE TECAN EVO 75

6.1 Follow the preventative maintenance procedure below as needed or before running case work.

6.1.1 Thoroughly flush the system with deionized water from the system fluid container.

6.1.2 Remove racks from the instrument surface. Carefully clean the work surface using a chloride disinfectant wipe and a KimWipe.

6.1.3 Empty all waste containers.

6.1.4 Clean the Teflon sample tip by gently wiping it with a lint-free tissue and deionized water.

6.1.5 Check the level of the system fluid container. It is good practice to replace system liquid at least once a week and to keep container filled.

6.1.6 Check the syringes for leaks, bubbles or internal contamination. If required, clean the syringes, taking care in removing syringes. If the syringes are leaking, replace the caps on the syringe plungers.

6.1.7 Check around the valve for signs of moisture.

6.1.8 Check the green Teflon coating of the stainless steel pipette tip for any damage.

6.1.9 Check for air bubbles or contamination in the pipetting tubing. Tighten the tubing connections or replace the tubing as required.

6.1.10 Clean the toothed rack with a lint-free tissue.

6.1.11 Clean the system liquid container with a mild soap. Make sure to thoroughly rise out container before filling with deionized water.

6.2 Acid-Base Wash

6.2.1 Performing an Acid-Base wash using 1N HCl and 1N NaOH is recommended for the maintenance of the Tecan Evo 75. This procedure can be done as needed.

6.2.2 Double click on the "NaviTrak2 Shortcut" icon.

6.2.3 Click "Start."

6.2.4 Click on the "Utilities" tab.

6.2.5 Click the "Acid/Base Wash" button.

6.2.6 Click the "Run" button and follow the pop-up window instructions.

6.2.7 Document in the maintenance log.

## 7 REFERENCES

7.1 TECAN Operating Manual Freedom EVO 75 BG/N: 30023958.02.

7.2 TECAN Freedom Evo 75, Operators Manual. Chapter 7 Preventive Maintenance and Repairs.

7.3 Enzyme-Linked Immunosorbent Assay (ELISA), Standard Operating Procedure.

7.4 Operation and Maintenance of the Plate Washer, Standard Operating Procedure.

7.5 Operation and Maintenance of the Plate Reader, Standard Operating Procedure.

7.6 LAB Form-61, EIA Maintenance Log.

7.7 Lab From-62, EIA Troubleshooting Log.

# Operation and Maintenance of the TECAN HydroFlex Plate Washer

1 **PURPOSE**

The TECAN Hydroflex Plate Washer is used to rinse microtiter plates that are used for drugs-of-abuse screening by Enzyme- Linked Immunosorbent Assay ("ELISA").

2 **SCOPE**

This document describes the operation and maintenance of the TECAN HydroFlex. Further operational instruction is found in the ELISA SOP.

3 **EQUIPMENT**

TECAN HydroFlex Plate Washer

4 **REAGENTS AND MATERIALS**

Deionized Water

5 **PROCEDURE**

5.1 Switch on the plate washer.

5.2 Use the ">" key to select between options, "OK" to confirm, and "□" to go back.

5.3 Priming: Select "Procedures", then "Prime". Select "Time10 sec" and press "OK" to start priming. The system must be primed with the liquid that will be used to wash the plates. Repeat the priming steps several times.

5.4 After the system is primed, place the microplate to be tested into the corresponding slot in the instrument.

5.5 From "Program", select "Start" and then select "ELISA". Press OK on "Run 1: ELISA".

5.6 Enter the number of strips on the plate. The plate washer washes 2 strips at a time. "S" denotes the presence of a strip and "N" denotes no strip. Example: If 4 strips need to be washed, the screen display should be 'SSSSNNNNNNNN'.

5.7 Click "OK".

5.8 The plate washer washes the wells 6 times.

5.9 After washing is complete, place the plates back into its respective slot on the TECAN EVO 75 instrument.

6 **MAINTENANCE AS NEEDED**

6.1 Rinsing – Rinsing is performed to flush the liquid system and to prevent needle blockages. During the rinse procedures, the needles are soaked in the prime tray.

6.2 Rinse Menu – The rinse menu has the following options:

6.2.1 Rinse Day – This is done if the instrument is to be left to stand for a short time of up to two hours. Rinse day can be performed with wash buffer or deionized water.

6.2.1.1 Procedure

6.2.1.1.1 Switch on the Plate Washer.
6.2.1.1.2 Press “>” key to find Procedures.
6.2.1.1.3 Press OK on Procedures.
6.2.1.1.4 Select “Rinse”.
6.2.1.1.5 Press OK to enter the Rinse menu.
6.2.1.1.6 Select “Rinse Day” procedure.
6.2.1.1.7 Confirm that the rinsing solution and the tube connecting the solution to the plate washer are connected.
6.2.1.1.8 Confirm waste bottle is empty.
6.2.1.1.9 Select “channel 1”. The tube connecting the plate washer to the rinse bottle has the number on it.
6.2.1.1.10 Select the duration time or the volume.
6.2.1.1.11 Once the rinse procedure is complete, press “stop” to abort the rinse procedure.
6.2.1.1.12 END appears on the display when the procedure is completed. The manifold remains immersed in the prime tray until END is pressed. Press END to finish the rinse procedure and the firmware returns to the Program menu.

6.2.2 Rinse Night –the instrument is left to stand 12 hours or longer with the manifold soaking in deionized water. Rinse night must be performed with deionized water only.

6.2.2.1 Procedure

6.2.2.1.1 Switch on the Plate Washer.
6.2.2.1.2 Press “>” key to find Procedures.
6.2.2.1.3 Press “OK” on Procedures.
6.2.2.1.4 Select “Rinse”.
6.2.2.1.5 Press OK to enter the Rinse menu.
6.2.2.1.6 Select “Rinse Night” procedure.
6.2.2.1.7 Confirm that the rinsing solution and the tube connecting the solution to the plate washer are connected.
6.2.2.1.8 Confirm waste bottle is empty.
6.2.2.1.9 Select “channel 1”. The tube connecting the plate washer to the rinse bottle has the number on it.
6.2.2.1.10 Select “OK” and the wash system is rinsed.
6.2.2.1.11 The prime tray is filled with liquid and the manifold is lowered into it.
6.2.2.1.12 Once the rinse procedure is complete, press “stop” to abort the rinse procedure.
6.2.2.1.13 Do not switch off the instrument.
6.2.2.1.14 END appears on the display when the procedure is completed. The manifold remains immersed in the prime tray until END is pressed. Press END to finish the rinse procedure and the firmware returns to the Program menu.
6.2.2.1.15 In case of instrument malfunction, inform Laboratory Management immediately and document the problem on lab form LAB-62 (EIA Troubleshooting Log) or use an equivalent method. Take necessary corrective action.

## 7 REFERENCES

7.1 TECAN HydroFlex Plate Washer Operating Manual, Document Part No: 30026397, 2008-02
7.2 TX-03-01 (Drugs of Abuse Screening by ELISA), Standard Operating Procedure.
7.3 TX-07-03 (Operation and Maintenance of the Plate Reader), Standard Operating Procedure.
7.4 Lab Form-61, EIA Maintenance Log.
7.5 Lab Form-62, EIA Troubleshooting Log.

# Operation and Maintenance of the TECAN Sunrise Plate Reader

## 1 PURPOSE

The TECAN Sunrise Plate Reader is a spectrophotometer, used to measure absorbance of 96-well microtiter plates in the Enzyme- Linked Immunosorbent Assay ("ELISA").

## 2 SCOPE

This document describes the operation and maintenance of the TECAN Sunrise plate reader. Further operational instruction is found in the ELISA SOP.

## 3 EQUIPMENT

TECAN Sunrise Plate Reader

## 4 PROCEDURE

4.1 Double click on Magellan 7.2 icon on the desk top.
4.2 Click the Start Measurement Wizard.
4.3 Click on the green button.
4.4 Click the Use Predefined Method
4.5 Click on blood or urine folder depending on the samples.
4.6 Click on the respective plate that needs to be read. (e.g., thc_b.mth for THC blood samples. Similarly, THC_U.mth for urine samples.)
4.7 Verify that the date is correct in the workspace.
4.8 Verify the correct method is selected.
4.9 Under the Sample ID List, click on "Insert".
4.10 Click on "import".
4.11 Select "Tecan-Files" from the files drop down box.
4.12 Select the microplate that needs to be read. Click on "open".
4.13 The plate map with the list of samples that has been loaded in the racks is displayed.
4.14 Click "OK".
4.15 Click on "Start". The UV spectrophotometer measures the absorbance at two wavelengths, 450nm and 620nm.
4.16 Click on "Finish".
4.17 Click "Save" to save the data.
4.18 In case of instrument malfunction, inform Laboratory Management immediately and document the problem on the lab form LAB-62 (EIA Troubleshooting Log) or using an equivalent method. Take any necessary correction action.
4.19 **Note**: For further instructions on the Sunrise Absorbance Reader, refer to the operating manual "TECAN instructions for the Use of Sunrise Absorbance Reader + Option Touchscreen Color".

## 5 REFERENCES

5.1 TECAN Sunrise Plate Reader Operating Manual, Document Part No.: 30041769, 2008-11; Document Revision No.: 1.5.

5.2 TX-03-01 (Drugs of Abuse Screening by ELISA), Standard Operating Procedure.
5.3 TX-07-02 (Operation and Maintenance of the Plate Washer), Standard Operating Procedure.
5.4 Lab form LAB-62, EIA Troubleshooting Log.

# Enzyme-Linked Immunosorbent Assay (ELISA)

1 **PURPOSE**

Preliminary screening of blood or urine samples for certain drugs or classes of drugs may be performed using Enzyme-Linked Immunosorbent Assay (ELISA). ELISA relies on drug-specific antibodies which are attached to polystyrene wells on a 96 well microliter plate. The unknown sample is fortified to the plate with a drug-enzyme conjugate (horseradish peroxidase). Any free drug in the sample competes with the conjugate for antibody binding sites on the surface of the well. After the well is washed, a chromogenic substrate is added, and a color is produced by catalysis by horseradish peroxidase. The enzymatic reaction is stopped by using dilute hydrochloric acid and then the absorbance is measured at 450 and 620 nm. The intensity of the color is inversely proportional to the concentration of drug in the sample.

2 **SCOPE**

This protocol describes usage of ELISA as a preliminary, qualitative screen for certain drugs or classes of drugs.

3 **REAGENTS AND KITS (SUPPLIED BY THE VENDOR)**

3.1 Antibody coated polystyrene microliter plates. These are purchased as commercial kits (Immunalysis Corporation).

3.1.1 Cannabinoids (THCA) Direct ELISA Kit

3.1.2 Cocaine Metabolite (BE Specific) Direct ELISA Kit

3.1.3 Opiates Direct ELISA Kit

3.1.4 PCP Direct ELISA Kit

3.1.5 Methamphetamine Direct ELISA Kit

3.1.6 Benzodiazepines Direct ELISA Kit

3.2 Enzyme conjugate: Horseradish peroxidase labeled drug and diluted in a protein matrix with protein stabilizers.

3.3 Substrate Reagent: Each bottle contains 3, 3', 5, 5'- tetramethylbenzidine and urea peroxidase in buffer.

3.4 Stop Reagent: Each bottle contains 1N hydrochloric acid (HCl).

3.5 Forensic Diluent (pH 7.0): Phosphate buffered saline (PBS) consisting of 150 mM saline in 100 mM phosphate buffer.

3.5.1 PBS may be prepared in-house:

3.5.1.1 Add 7.0 g of dibasic sodium phosphate to a 500 mL volumetric flask and bring to volume with deionized water while stirring.

3.5.1.2 In a second 500 mL volumetric flask, add 6.0 g of monobasic sodium phosphate and bring to volume with deionized water while stirring.

3.5.1.3 Mix the two solutions together until a pH of 7.0 is obtained.

3.5.1.4 Add sufficient sodium chloride to bring the concentration to 150mM.

3.5.1.5 For 1 L phosphate buffer, add 8.8 g sodium chloride.

3.5.1.6 Store refrigerated (expiration 6 months).

3.6 EIA Stock standards for blood and urine: 1 mg/mL in methanol.
3.7 **NOTE**: Allow all reagents to come to room temperature before use.

## 4 STANDARDS

4.1 EIA Urine Drug Standard Mix:
4.1.1 Using methanolic drug standards, in a 10 mL volumetric flask add the following:
4.1.1.1 100 µL of 0.1 mg/mL *l*-11-nor-9-carboxy-9-tetrahydrocannabinol (THCA),
4.1.1.2 1000 µL of 0.1 mg/mL *d*-methamphetamine,
4.1.1.3 1500 µL of 0.1 mg/mL benzoylecgonine (BE),
4.1.1.4 500 µL of 0.1 mg/mL oxazepam,
4.1.1.5 1000 µL of 0.1 mg/mL morphine, and
4.1.1.6 125 µL of 0.1 mg/mL phencyclidine (PCP).
4.1.2 Bring to volume with methanol. Store in a silanized glass vial in the refrigerator (6 months expiration).
4.2 EIA Blood Drug Standard Mix:
4.2.1 Using methanolic drug standards, in a 25 mL volumetric flask add the following:
4.2.1.1 100 µL of 0.1 mg/mL of *l*-THCA,
4.2.1.2 200 µL of 0.1 mg/mL of *d*-methamphetamine,
4.2.1.3 500 µL of 0.1 mg/mL of benzoylecgonine (BE),
4.2.1.4 500 µL of 0.1 mg/mL of oxazepam,
4.2.1.5 200 µL of 0.1 mg/mL of morphine, and
4.2.1.6 100 µL of 0.1 mg/mL of phencyclidine (PCP).
4.2.2 Bring to volume with methanol. Store in a silanized glass vial in the refrigerator (6 months expiration).

## 5 EQUIPMENT

5.1 TECAN Freedom Evo 75
5.2 TECAN HydroFlex Plate Washer
5.3 TECAN Sunrise Plate Reader
5.4 Vortex mixer
5.5 Air Displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)
5.6 Centrifuge
5.7 Sonicator
5.8 Homogenizer

## 6 PROCEDURE FOR BLOOD AND URINE

6.1 In each run, the following controls are included:
6.1.1 Positive control (in duplicate) – fortified with target drugs at the specified threshold.
6.1.2 Negative control (in duplicate) – drug free matrix.
6.1.3 Low QC (in duplicate) – fortified with target drugs at half the concentration found in the positive control.
6.1.4 High QC (in duplicate) – fortified with target drugs at five times the concentration found in the positive control.

6.2 Positive Control Concentrations

6.2.1 Samples with absorbance readings BELOW the positive control are considered **PRESUMPTIVE POSITIVE. Confirmatory analysis is routinely indicated for all presumptive positive cases.**

6.2.2 **Note:** Confirmatory analysis may be requested on any sample, regardless of preliminary screening results.

| Positive Control | Blood (ng/mL) | Urine (ng/mL) |
|---|---|---|
| oxazepam | 50 | 100 |
| *l*-THCA | 10 | 20 |
| benzoylecgonine | 50 | 300 |
| Phencyclidine | 10 | 25 |
| d-methamphetamine | 20 | 200 |
| morphine | 20 | 200 |

Preparation of QCs:

6.2.3 Blood QCs (**PREPARED DAILY FROM THE BLOOD DRUG STANDARD**) using drug-free blood preserved with 1% NaF (Sodium Fluoride) and 0.2% K2C2O4 (Potassium Oxalate) proceed as follows:

6.2.3.1 High QC: To 1.0 mL of drug-free blood, add 125 µL of Blood Drug Standard Mix

6.2.3.2 Positive Control: To 1950 µL of drug-free blood, add 50 µL of Blood Drug Standard Mix.

6.2.3.3 Low QC: To 1975 µL of drug-free blood, add 25 µL of Blood Drug Standard Mix.

| Assay | Positive Control (ng/mL) | Low QC (ng/mL) | High QC (ng/mL) | Negative Control (ng/mL) |
|---|---|---|---|---|
| Oxazepam | 50 | 25 | 250 | 0 |
| *l*-THCA | 10 | 5 | 50 | 0 |
| Benzoylecgonine | 50 | 25 | 250 | 0 |
| Phencyclidine | 10 | 5 | 50 | 0 |
| *d*-methamphetamine | 20 | 10 | 100 | 0 |
| Morphine | 20 | 10 | 100 | 0 |

6.2.4 Urine QCs (**PREPARED DAILY FROM THE URINE DRUG STANDARD**)

Using drug-free urine preserved with 1% NaF (Sodium Fluoride) proceed as follows:

6.2.4.1 High QC: To 900 µL drug-free urine, add 100 µL of Urine Drug Standard Mix.

6.2.4.2 Positive Control: To 1960 µL of drug-free urine, add 40 µL of Urine Drug Standard Mix.

6.2.4.3 Low QC: To 1980 µL of drug-free urine, add 20 µL of Urine Drug Standard Mix.

| Assay | Positive Control (ng/mL) | Low QC (ng/mL) | High QC (ng/mL) | Negative Control(ng/mL) |
|---|---|---|---|---|
| oxazepam | 100 | 50 | 500 | 0 |
| *l*-THCA | 20 | 10 | 100 | 0 |
| benzoylecgonine | 300 | 150 | 1500 | 0 |
| Phencyclidine | 25 | 12.5 | 125 | 0 |
| *d*-methamphetamine | 200 | 100 | 1000 | 0 |
| morphine | 200 | 100 | 1000 | 0 |

6.3 Subsampling:

6.3.1 Blood 1:10 dilution with forensic diluent (phosphate buffered saline)

6.3.1.1 Pipette 100µL of controls, negative (blood), and the unknowns into subsample tubes.

**Note**: If blood samples are clotted, remove clots and decant sample into new subsample tube. Sonication, centrifugation, or other sample preparation steps may be necessary for degraded specimens.

6.3.1.2 Add 900 µL of Forensic diluent (PBS buffer) to each subsample tube and vortex.

6.3.2 Urine 1:10 dilution with forensic diluent (phosphate buffered saline)

6.3.2.1 Pipette 100µL of controls, negative (urine), and the unknowns into subsample tubes.

**Note**: If samples contain particulate material, centrifugation may be performed before transfer into the subsample tube.

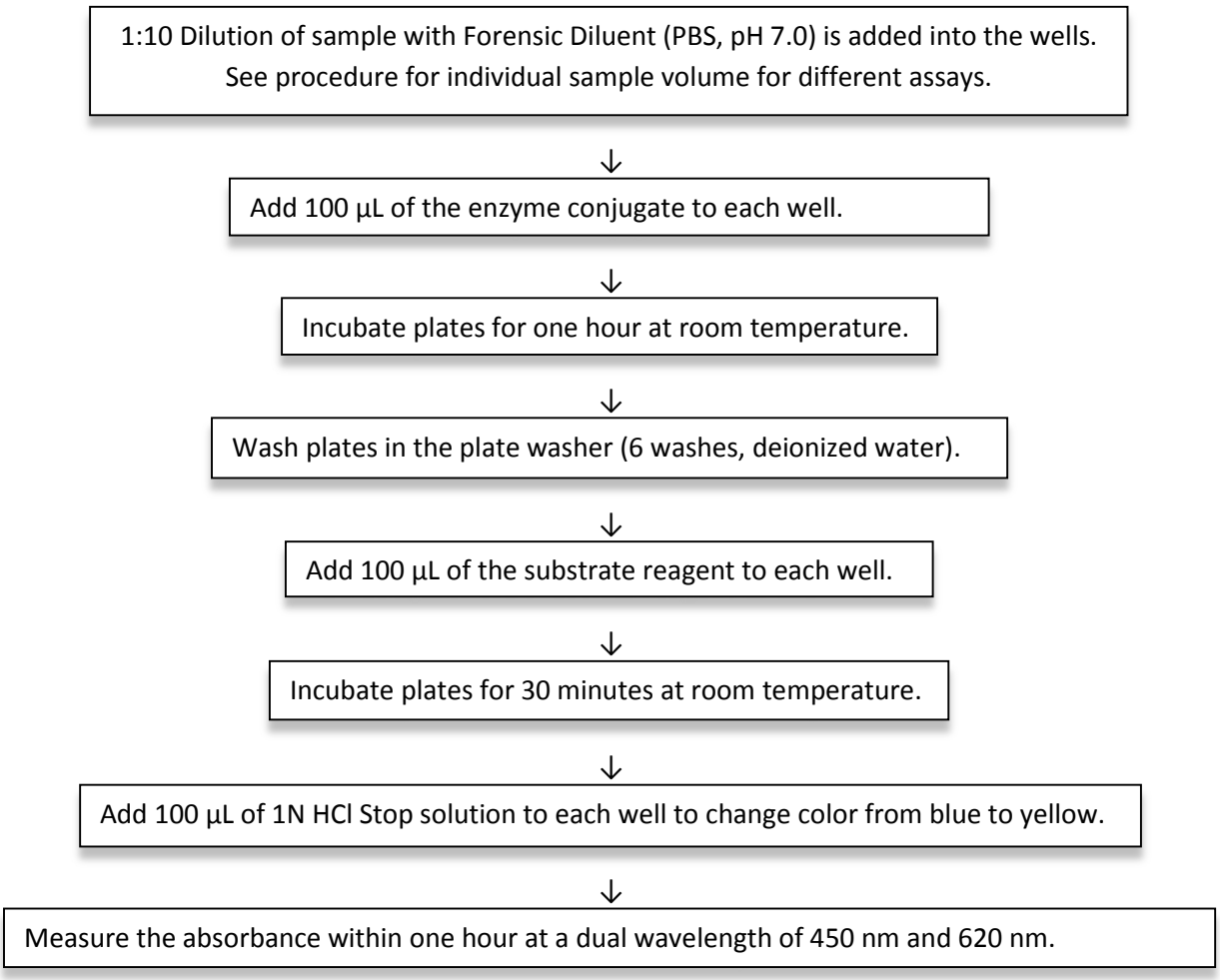
6.3.2.2 Add 900 µL of Forensic diluent (PBS buffer) to each subsample tube and vortex.

6.4 Placement of samples

Place unknowns and QCs in appropriate positions in subsample racks (see chart below). **NOTE**: A High QC **must be run** in between every ten case samples and at the end of every run).

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | NEG | SAMPLE | SAMPLE | SAMPLE | SAMPLE | HIGH QC |
| B | NEG | SAMPLE | SAMPLE | SAMPLE | SAMPLE | SAMPLE |
| C | POS QC | SAMPLE | HIGH QC | SAMPLE | SAMPLE | SAMPLE |
| D | POS QC | SAMPLE | SAMPLE | SAMPLE | SAMPLE | SAMPLE |
| E | LOW QC | SAMPLE | SAMPLE | SAMPLE | SAMPLE | SAMPLE |
| F | LOW QC | SAMPLE | SAMPLE | HIGH QC | SAMPLE | SAMPLE |
| G | HIGH QC | SAMPLE | SAMPLE | SAMPLE | SAMPLE | SAMPLE |
| H | HIGH QC | SAMPLE | SAMPLE | SAMPLE | SAMPLE | HIGH QC |

## 7 FLOW CHART


1:10 Dilution of sample with Forensic Diluent (PBS, pH 7.0) is added into the wells. See procedure for individual sample volume for different assays.
Add 100 µL of the enzyme conjugate to each well.
Incubate plates for one hour at room temperature.
Wash plates in the plate washer (6 washes, deionized water).
Add 100 µL of the substrate reagent to each well.
Incubate plates for 30 minutes at room temperature.
Add 100 µL of 1N HCl Stop solution to each well to change color from blue to yellow.
Measure the absorbance within one hour at a dual wavelength of 450 nm and 620 nm.

7.2 **To edit or create a method:**

7.2.1 Go to the main menu

7.2.2 Click on “create/edit a method.”

7.2.3 Click “OK.”

7.2.4 Click “open.”

7.2.5 Click on the ELISA kit test number

7.2.6 Click on “Endpoint measurement.”

7.2.7 Click on “define evaluation.” The plate map is displayed.

7.2.8 Click on the boxes that need to be changed.

7.2.9 Select the identifiers Acronym.

7.2.10 Click “Fill Selection.”

7.2.11 Click “Finish.”

7.2.12 Click “Save.”

7.3 The instrument dispenses the following volumes for blood assays:

| Assay | Volume Blood (µL)* | Volume Conjugate (µL) | Volume TMB Substrate (µL) | Volume Stop Solution (µL) |
|---|---|---|---|---|
| oxazepam | 50 | 100 | 100 | 100 |
| *l*-THCA | 50 | 100 | 100 | 100 |
| benzoylecgonine | 50 | 100 | 100 | 100 |
| Phencyclidine | 20 | 100 | 100 | 100 |
| *d*-methamphetamine | 50 | 100 | 100 | 100 |
| morphine | 20 | 100 | 100 | 100 |

*Diluted matrix (1:10 in PBS or Forensic Diluent)

7.4 The instrument dispenses the following volumes for urine assays:

| Assay | Volume Urine (µL)* | Volume Conjugate (µL) | Volume TMB Substrate (µL) | Volume Stop Solution (µL) |
|---|---|---|---|---|
| oxazepam | 50 | 100 | 100 | 100 |
| *l*-THCA | 50 | 100 | 100 | 100 |
| benzoylecgonine | 10 | 100 | 100 | 100 |
| Phencyclidine | 10 | 100 | 100 | 100 |
| *d*-methamphetamine | 10 | 100 | 100 | 100 |
| morphine | 10 | 100 | 100 | 100 |

*Diluted matrix (1:10 in PBS or Forensic Diluent)

7.5 Samples incubate with the drug-enzyme conjugate for 1 hour and with the enzyme substrate for 30 minutes.

7.6 The EIA operator is responsible for completing EIA QC Logs (for blood and urine), EIA batch reports, and submitting data for review. Once reviewed, EIA results are placed in folders and depending on the results, requests for confirmatory analysis are placed.

## 8 OTHER MATRICES

8.1 Appropriate sample preparation steps must be used for tissues or other alternative matrices that may be encountered. Tissues are routinely homogenized using deionized water (1:1) prior to analysis. Additional water may be added to achieve full homogenization. Document the conditions for homogenization in the case folder. Following homogenization, tissues and solids are analyzed in accordance with blood samples.

8.2 Sample preparation for alternative matrices may include homogenization, filtration, sonication, centrifugation, or dilution. Liquid-based alternative matrices are analyzed in accordance with urine samples.

8.3 Document all sample preparation steps in the case folder.

## 9 INTERPRETATION OF RESULTS

9.1 All samples with absorbance readings BELOW the positive control (mean of duplicate analysis) are presumptive positive.

9.2 Confirmatory analyses are requested on all presumptive positive samples unless specifically directed otherwise by the submitting agency or client.

9.3 Results are qualitative in nature. Quantitative results are not possible due to the non- linear binding characteristics of the assay and antibody specificity.

9.4 EIA reports display a numerical value for all cases, calculated as follows:

9.4.1 (A positive control /A sample) x C positive control

9.4.2 Example:

9.4.3 A case sample produces an absorbance reading of 0.5 for the cannabinoid assay (A sample =0.5); the mean absorbance for the positive control is 1.0 (A positive control =1.0).

9.4.4 The concentration of *l*-THCA in the positive control is 10 ng/mL (C positive control=10), so the numerical value assigned to the sample is (1.0/0.5) x 10 = 20 ng/mL.

9.4.5 Note that these numerical values are not quantitative, but can be used to differentiate low and high concentrations in case samples. These values should be considered when performing confirmatory analyses.

## 10 QUALITY ASSURANCE

10.1 EIA QC Logs

10.1.1 After each run, the following data is entered into the Blood EIA QC Log or Urine EIA QC Log:

10.1.1.1 Mean absorbance for the positive control

10.1.1.2 Mean % Binding for the positive control

10.1.1.3 CV (%) for duplicate analyses for the positive control

10.1.1.4 Mean absorbance for the negative control

10.1.1.5 CV (%) for duplicate analyses for the negative control

10.1.1.6 (The mean % Binding for the negative control is 100% by default)

10.1.1.7 Mean absorbance for the High QC Mean % Binding for the High QC

10.1.1.8 Mean absorbance for the Low QC Mean % Binding for the Low QC

10.1.2 The spreadsheet calculates the running mean % Binding (for all assays), CVs, and displays results on a control chart for evaluation of trends.

% Binding is calculated numerically as follows:

$\%B = (A_{\text{positive control}}/A_{\text{negative control}}) \times 100$

Example:

A positive control produces a mean absorbance reading of 0.66 ($A_{\text{positive control}}$ =0.66); the mean absorbance for the negative control is 2.0 ($A_{\text{negative control}}$ =2.0). The % Binding is 0.66/2.0) x 100 = 33%

10.1.3 The control charts are reviewed with the EIA batch file. Only a copy of the EIA results is placed in the case folder. EIA results are reviewed and stored in batch files in the laboratory.

10.2 **Acceptance Criteria**

10.2.1 During technical review of the EIA batch file, the following acceptance criteria apply to all assays:

10.2.1.1 % Binding for the POSITIVE CONTROL must be < 60%, and

10.2.1.2 % CV for the NEGATIVE CONTROL (n=2) must be < 20%

10.2.2 Assays not meeting both of these criteria should be repeated. Repeat analysis may be warranted for other reasons. Variations in assay performance are expected between lot numbers. Changes in procedure may be necessary to maintain performance. Any changes are prominently documented in the EIA batch file.

10.2.3 The examiner and technical reviewer will also evaluate:

10.2.3.1 Overall reproducibility (poor reproducibility is typically due to liquid handling)

10.2.3.2 Lot specific changes to assays

10.2.3.3 Trends that are not assay specific (which can indicate liquid handling issues or the need for additional preventive maintenance, washing etc.)

10.2.3.4 Separation (absorbance difference) between the negative and positive controls

10.2.3.5 Absolute absorbance of the negative control (low absorbance may result in a high %CV)

10.2.3.6 Drift or reproducibility across the plate (evaluated using the High QC)

10.2.3.7 Responses of the Low QC and High QC

## 11 LITERATURE AND SUPPORTING DOCUMENTATION

11.1 Smith, M. Immunoassay in Principles of Forensic Toxicology, 3rd Edition. Levine B (Ed). AACC Press, Washington DC, 2009, pp 117-138.

11.2 Kerrigan, S and Phillips Jr., W.H. Comparison of ELISAs for Opiates, Methamphetamine, Cocaine Metabolite, Benzodiazepines, Phencyclidine and Cannabinoids in Whole Blood and Urine, Clin Chem: 47, 540-547 (2001).

11.3 TX-07-01 (Operation and Maintenance of Tecan Freedom Evo 75), Sam Houston State University Standard Operating Procedure.

11.4 TX-07-02 (Operation and Maintenance of Plate Washer), Sam Houston State University Standard Operating Procedure.

11.5 TX-07-03 (Operation and Maintenance of Plate Reader), Sam Houston State University Standard Operating Procedure.

11.6 TECAN Freedom Evo 75, Operators Manual.

11.7 TECAN HydroFlex Plate Washer, Operators Manual.

11.8 TECAN Sunrise Plate Reader, Operation Manual.

# Reagents for Drug Screening/Confirmation Analyses

1 **PURPOSE**

This document outlines the preparation of reagents used in drug screening/confirmation analysis.

2 **SAFETY**

This procedure must be conducted in accordance with the HFSC Health and Safety Manual and Quality Manual. All case specimens should be treated with universal blood borne pathogen precautions. Appropriate personal protective equipment must be worn during sample and reagent preparation and when handling caustic chemicals. Flammable liquids and vapors may cause eye, skin and respiratory tract irritation. Derivatization reagents are toxic and must be handled in a chemical safety hood or well-ventilated area. Material Safety Data Sheets (MSDS) are available in the laboratory.

3 **REAGENTS**

Organic solvents are HPLC grade or higher and inorganic reagents (e.g. salts, acids) are ACS grade. Deionized water is prepared using a Millipore Direct Q UV3 water system (or equivalent). Preparations of reagents are documented in a retrievable format.

3.1 Glacial acetic acid
3.2 Hydrochloric acid
3.3 Ammonium hydroxide
3.4 Dibasic sodium phosphate
3.5 Methylene chloride
3.6 Isopropanol
3.7 Methanol
3.8 Ethyl acetate
3.9 Deionized (DI) water [7732-18-5]

4 **1 M Acetic acid**

4.1 Add 28.6 mL of concentrated glacial acetic acid to a 500 mL volumetric flask half-filled with deionized water. Bring to volume with deionized water and mix.
4.2 Storage: Room temperature
4.3 Discard: 1 year

5 **100 mM, pH 6.0 Phosphate buffer**

5.1 Add 7.0 g of dibasic sodium phosphate to a 500 mL volumetric flask and bring to volume with deionized water while stirring. In a second 500 mL volumetric flask, add 6.0 g of monobasic sodium phosphate and bring to volume with deionized water while stirring. Mix the two solutions together until a pH of 6.0 is obtained.
5.2 Storage: Refrigerated
5.3 Discard: 6 months

6 **100 mM sodium phosphate buffer, pH 6.8 – Deconjugation Buffer**

6.1 Add 7.0 g of dibasic sodium phosphate to a 500 mL volumetric flask and bring to volume with deionized water while stirring. In a second 500 mL volumetric flask, add 6.0 g of monobasic sodium phosphate and bring to volume with deionized water while stirring. Mix the two solutions together until a pH of 6.8 is obtained
6.2 Storage: Refrigerated
6.3 Discard: 6 months

7 **80:20 Methylene Chloride/isopropanol**
7.1 In a 500 mL volumetric flask, add 100 mL of isopropanol to 400 mL of methylene chloride.
7.2 Storage: Room temperature
7.3 Discard: 6 months

8 **2% Ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol – Elution Solvent**
8.1 In a 25 mL volumetric flask, add 0.5 mL concentrated ammonium hydroxide. Bring to volume with 80:20 methylene chloride/isopropanol.
8.2 Storage: Room temperature
8.3 Discard: after each daily use

9 **2% Ammonium hydroxide (concentrated) in ethyl acetate with 1 mL of methanol**
9.1 In a 50 mL volumetric flask partially filled with ethyl acetate, add 0.5 mL concentrated ammonium hydroxide. Bring volume to 25 mL with ethyl acetate. Add 1 mL of Methanol. Mix vigorously.
9.2 Storage: Room temperature
9.3 Discard: after each daily use

10 **1% Hydrochloric acid in methanol – Acidic Methanol**
10.1 Add 1 mL of concentrated hydrochloric acid into a 100 mL volumetric flask using a volumetric pipette. Bring to volume with methanol.
10.2 Storage: Room temperature
10.3 Discard: 3 months

11 **0.1 M Hydrochloric acid**
11.1 Add 8.4 mL concentrated hydrochloric acid to a 1 liter volumetric flask containing deionized water. Bring to volume with deionized water.
11.2 Storage: Room temperature
11.3 Discard: 12 months

12 **1 M Hydrochloric acid**

12.1 Add 84.6 mL concentrated hydrochloric acid to a 1 liter volumetric flask containing deionized water. Bring to volume with deionized water.
12.2 Storage: Room temperature
12.3 Discard: 12 months

# Evaluation of Results from Gas Chromatography/Mass Spectroscopy

## 1 PURPOSE

The GC/MS is composed of two major building blocks: the gas chromatograph (GC) and the mass spectrometer (MS). The GC utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the stationary phase properties. The difference in the chemical properties of the different compounds in a sample and their relative affinity for the stationary phase of the column will promote separation of these compounds as the sample travels through the length of the column. The compounds are retained by the column and then elude from the column at different times (retention time). The mass spectrometer is the detector portion of the instrument. As the compounds elude off the column and enter into the MS, they are ionized, fragmented, separated and detected based upon their mass-to-charge ratio (m/z). The results obtained from GC/MS analysis must be evaluated to determine the acceptability of the results. This applies to both quantitative and qualitative results.

## 2 SCOPE

This policy applies to all GC/MS analysis.

## 3 QUALITY CONTROLS

3.1 Positive and negative QCs must be included in each run. Each run must end with a negative and positive control. For qualitative analysis each batch must contain a negative (drug-free) extract and an extracted drug standard.

3.2 For quantitative analysis, an in-house or external QC must be included. Record the calculated concentrations in the appropriate QC Log.

## 4 BLOOD AND URINE

Blood is the preferred specimen for impairment cases as it reveals what compounds may be affecting the individual and the concentration of the compounds may be associated with impairment. Urine is an acceptable specimen but is typically not quantitated due to variations in volume.

## 5 TISSUES AND OTHER MATRICES

5.1 Appropriate sample preparation steps must be used for tissues or alternative matrices that may be encountered. Tissues are routinely homogenized using deionized water (1:1) prior to analysis. Additional water may be added to achieve full homogenization. Document the conditions for homogenization in the case folder. Following homogenization, tissues, and other solids are analyzed in accordance with blood samples.

5.2 Sample preparation for alternative matrices may include homogenization, filtration, sonication, centrifugation, and/or dilution. Liquid-based alternative matrices are analyzed in accordance with urine samples.

5.3 DOCUMENT ALL SAMPLE PREPARATION STEPS IN THE CASE RECORD AND INCLUDE APPROPRIATE MATRIX MATCHED CONTROLS.

## 6 QUALITATIVE ANALYSIS

6.1 All mass spectral libraries and/or reference spectra must be approved by the Technical Leader or Toxicology Manager.

6.2 The following acceptance criteria may be used as guidelines, *but not as absolutes*, to identify a substance through its mass spectrum:

6.2.1 Usually the base peak will be the same and isotope clusters will contain the same major ions and relative abundances.

6.2.2 When compared to a reference spectrum, the spectra must contain strong similarities.

6.2.3 Any differences between the reference spectrum and the unknown spectrum must be carefully evaluated for acceptability.

6.3 The following criteria **must be met** for qualitative identification of a compound:

6.3.1 Retention times (RT) and/or relative retention times (RRT) must be within ± 2% of the target.

6.3.2 Case mass spectrum must be of comparable quality to a reference spectrum in mass assignment and intensity.

6.3.3 Any extraneous ions of significant intensity that are not found in the reference spectrum must be demonstrated as being from background or known interference by using an extracted ion chromatogram.

6.4 Qualitative identification is determined using characteristic retention time and mass spectral characteristics. Deuterated internal standards in each sample are used to identify the correct retention time of the drug. For SIM, all three characteristic ions must be present. Ion intensity and ratios should be taken into consideration. Ion ratios should be within +/- 20% of the calibrator or the QC. These are evaluated on a case by case basis and are subjected to technical and administrative review.

## 7 QUANTITATIVE ANALYSIS

7.1 Concentration of a drug is determined using linear regression analysis. At least 3 calibrators and a blank blood sample must be used for each quantitation. $R^2$ values of at least 0.99 should be attained following linear regression analysis. If more than three calibrators are included in the run, one may be excluded if the response factor is not within ± 20% of the remaining calibrators. The response factor is defined as follows: (Peak Area of the Internal Standard/Peak Area of the Calibrator) x Calibrator Concentration. If the response factor is within range, it may be permissible to exclude the point from the calibration provided that the independent control is still within acceptable limits and the reviewer is in agreement. The calculated concentration of a calibrator must be within ± 20% of the expected value. When determining the acceptability of a calibrator, the concentration range is taken into account. If a calibrator needs to be excluded from the curve, the action should be described and justified in the Batch file.

7.2 Quantitative values above or below the highest and lowest calibrators should be reported as greater than or less than the appropriate calibrator concentration. If the calculated concentration is within ± 20% of either the lowest or highest calibrator, the result may be reported upon approval from the Technical Leader or Toxicology Manager. Dilution of a specimen may be necessary for samples that contain an elevated concentration of drug. Any

changes in sample volume that deviate from the technical procedure must be documented in the case folder.

7.3 For quantitative reporting, the calculated value of the in-house or external QC should be within ± 20% of the target concentration. Alternatively, quantitative results may be reported if the QC is ± 3 SD of the established mean.

## 8 TOXICOLOGY REPORTING GUIDELINES

8.1 Qualitative vs. Quantitative Results

8.1.1 Drugs in urine are reported qualitatively unless specifically noted otherwise in the standard operating procedure. Quantitative drug determinations in urine cannot be interpreted pharmacologically due to differences in total urine volume and elimination rate.

8.1.2 Unless specifically requested by the client, it is the policy of the laboratory not to report certain substances that are routinely encountered in toxicological specimens including caffeine, nicotine, and their metabolites and byproducts.

8.1.3 Blood is the preferred specimen for quantitative drug determination. Blood drug results may be reported either qualitatively or quantitatively. The laboratory reserves the right to determine whether qualitative or quantitative results are reported as determined by:

8.1.3.1 Class of drug

8.1.3.2 Case type/offense type

8.1.3.3 Other toxicological findings

8.1.3.4 Data and/or specimen quality

8.1.3.5 Quantity of sample

8.1.3.6 Technical abilities of the laboratory

8.2 In general, it is the policy of the laboratory not to perform quantitative analyses that have no interpretive value. For example, drugs or drug classes that are non-impairing (e.g. fluoxetine) are reported only qualitatively. Qualitative reports may be issued if insufficient sample exists for quantitation. Qualitative results may also be reported if the quantity of drug is very low (e.g., between the limit of detection and the limit of quantitation) or determined to have little importance compared to other results. The laboratory may only report qualitative and/or quantitative results for procedures demonstrated to be scientifically valid.

8.3 Screening and Confirmatory Tests

8.3.1 It is the policy of the laboratory not to report positive immunoassay screening results in the absence of confirmation. All presumptive positive drug test results by immunoassay are subject to confirmatory analysis using gas chromatography/mass spectrometry (GC/MS) or equivalent, provided there is sufficient specimen.

8.3.2 Preliminary screening tests such as immunoassay have limited scope and utility. They are predominantly used to direct the scope of other analytical testing. Immunoassay results are not reported unless they are negative and no additional tests are performed. In those instances, the report clearly states the scope of testing was limited to immunoassay and lists the drug classes and appropriate cutoffs.

# Alprazolam Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for confirmatory analysis of alprazolam by solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS). Quantitative and/or qualitative analysis are performed using deuterated internal standard and selective ion monitoring (SIM) in electron ionization (EI) mode.

## 2 SCOPE

This procedure should be used for confirmatory analysis of alprazolam from toxicology specimens, typically blood. This procedure is recommended if quantitative analysis is desired. Qualitative results may also be reported. For routine full scan qualitative analysis, refer to Basic, Acidic and Neutral Drugs by GC/MS.

## 3 REAGENTS AND STANDARDS

3.1 Alprazolam Working Standard

3.1.1 10 ng/µL Alprazolam Working Standard: Add 100 µL of alprazolam (1.0 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.1.2 1 ng/µL Alprazolam Working Standard: Add 1 mL of 10 ng/µL working standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2 Alprazolam-$D_5$ Internal Standard Solution

3.2.1 10 ng/µL Alprazolam – $D_5$ STOCK Internal Standard: Add 1 mL of Alprazolam-$D_5$ (100 µg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (12 month expiration).

3.2.2 1 ng/µL Alprazolam – $D_5$ WORKING Internal Standard: Add 1 mL of Alprazolam-$D_5$ (10 ng/µL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.3 1M Acetic Acid

3.4 100 mM, pH 6.0 Phosphate Buffer

3.5 Methylene Chloride/Isopropanol (80:20)

3.6 Elution Solvent: 2% ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)

4.2 Cerex PolyChrom Clin II SPE columns

4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold

4.4 Pan balance

4.5 pH meter

4.6 Evaporator

4.7 Vortex mixer
4.8 Centrifuge
4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD
4.14 Stir plate and stir bars

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m DB-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.2 mL/min with an injection volume of 2 µL in split mode (10:1).

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 140 °C<br>Hold for 0.5 minutes<br>30 °C/min to 290 °C<br>Hold for 2.5 minutes<br>50 °C/min to 310 °C<br>Hold for 2.6 minutes |
| Total Run Time: | 11 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

**BLOOD, URINE AND OTHER MATRICES:**

6.1 In a round bottom glass culture tube, add 1 mL of specimen and 100 µL Working Internal Standard Solution (1 ng/µL) while vortex mixing (total concentration = 100 ng/mL). NOTE IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME, ADD BUFFER AS THE DILUENT AND DOCUMENT IN THE CASE RECORD.

6.2 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. A minimum of one negative and one positive control must be included in each run for qualitative analysis. For quantitative analysis, a minimum of one quantitative QC must be included in each run. An in-house whole blood QC of 100 ng/mL is recommended. Enter results into the appropriate QC Log after each run. Use appropriate external controls (e.g., UTAK Whole Blood Controls) when available.

6.3 Add 2 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex. Sonication and/or centrifugation may be used if necessary. Document any additional sample pretreatment in the case folder.
6.4 Place collection tubes or reservoir (for waste) into vacuum manifold rack or positive pressure apparatus.
6.5 Centrifuge samples for at least 10 minutes at 4000 rpm.
6.6 Pour sample onto Polychrom Clin II columns. Use sufficient vacuum/pressure to draw samples through the columns.
6.7 Wash column with 1 mL deionized water.
6.8 Remove waste tubes/reservoir and replace with fresh waste tubes/reservoir.
6.9 Wash column with 1 mL 1 M acetic acid.
6.10 Dry column on full vacuum/pressure for 5 minutes.
6.11 Wash column with 1 mL hexane.
6.12 Place conical collection tubes into the SPE manifold. If the Teflon inserts (vacuum manifold only) appear dirty, replace them.
6.13 Elute drug with 1 mL ethyl acetate.
6.14 Turn on vacuum or apply positive pressure for a few seconds to ensure all elution solvent has drained from the column.
6.15 Remove conical collection tubes and replace with waste tubes/reservoir.
6.16 Wash column with 1 mL methanol.
6.17 Dry column on full vacuum/pressure for 5 minutes.
6.18 Replace waste tubes/reservoir with conical collection tubes containing the acidic/neutral fraction. If the Teflon inserts (vacuum manifold only) appear dirty, replace them.
6.19 Elute drug with 1 mL elution solvent (2% ammonium hydroxide in methylene chloride/isopropyl alcohol 80:20). ELUTION SOLVENT MUST BE MADE FRESH.
6.20 Turn on vacuum or apply positive pressure for a few seconds to ensure all elution solvent has drained from the column.
6.21 Evaporate the combined basic and acidic/neutral fractions to dryness under nitrogen for approximately 10 minutes at 50-60°C. Reconstitute in 30 µL ethyl acetate, vortex, and transfer to autosampler vials.
6.22 Inject 2 µL onto the GC/MS using ALPRAZOLAM.M.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.

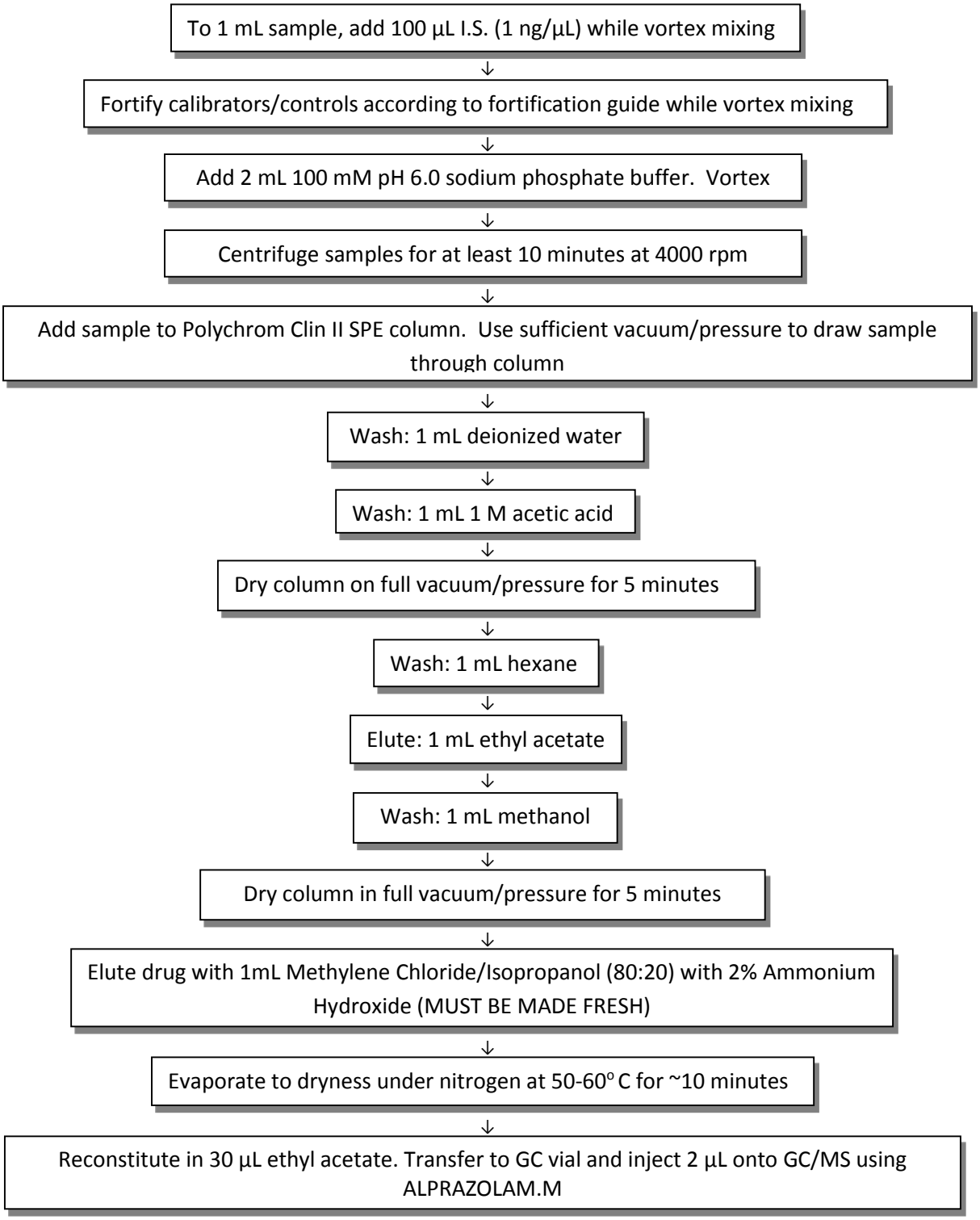
| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Alprazolam – D5 | 313 | 284, 278 | 9.09 |
| Alprazolam | 308 | 279, 273 | 9.10 |
| *Retention Time (RT) varies with column length. Deuterated (D5) internal standards are used throughout. Corresponding ions for internal standards are M+5. Refer to attached method for acquisition parameters (Alprazolam.M) | | | |

## 8 INTERPRETATION OF RESULTS

8.1 Assay performance including but not limited to precision, accuracy, limits of detection, quantitation, and linearity are summarized in the validation documentation.

8.2 The limit of detection in whole blood is 10 ng/mL and the limit of quantitation is 20 ng/mL.

## 9 FLOWCHART FOR ALPRAZOLAM ANALYSIS


To 1 mL sample, add 100 µL I.S. (1 ng/µL) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 2 mL 100 mM pH 6.0 sodium phosphate buffer. Vortex
Centrifuge samples for at least 10 minutes at 4000 rpm
Add sample to Polychrom Clin II SPE column. Use sufficient vacuum/pressure to draw sample through column
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes
Wash: 1 mL hexane
Elute: 1 mL ethyl acetate
Wash: 1 mL methanol
Dry column in full vacuum/pressure for 5 minutes
Elute drug with 1mL Methylene Chloride/Isopropanol (80:20) with 2% Ammonium Hydroxide (MUST BE MADE FRESH)
Evaporate to dryness under nitrogen at 50-60° C for ~10 minutes
Reconstitute in 30 µL ethyl acetate. Transfer to GC vial and inject 2 µL onto GC/MS using ALPRAZOLAM.M

## 10 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 20 | 1 | 20 |
| 1 | 50 | 1 | 50 |
| 1 | 100 | 1 | 100 |
| 1 | 250 | 10 | 25 |
| 1 | 500 | 10 | 50 |

## 11 LITERATURE AND SUPPORTING DOCUMENTATION

11.1 Cairns, Eric R., et. al. Quantitative Analysis of Alprazolam and Triazolam in Hemolysed Whole Blood and Liver Digest by GC/MS/NICI with Deuterated Internal Standards. J Anal Toxicol 1994 Jan-Feb; Vol. 18 (1), pp. 1-6.

11.2 Tiscione, Nicholas B., et. al. Quantitation of Benzodiazepines in Whole Blood by Electron Impact-Gas Chromatography-Mass Spectrometry. J Anal Toxicol 2008 Oct.; Vol. 32 (8), pp. 644-652.

11.3 Levine, Barry. Central Nervous System Depressants *in* Principles of Forensic Toxicology, 2nd Ed., Levine, Barry, Ed. AACC Press, Washington DC, pp 173-186 (2003).

11.4 Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 7th Ed., Biomedical Publications, Foster City, CA, p 35-36 (2004).

11.5 Method file: ALPRAZOLAM.M.

# Basic, Acidic and Neutral Drugs by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

Basic, acidic, and neutral drugs are extracted from the matrix using Cerex Polychrom Clin II solid phase extraction (SPE) columns and gas chromatography/mass spectrometry (GC/MS) analysis in electron ionization (EI) mode. This procedure is used to identify a wide range of basic, acidic, and neutral drugs. Mepivacaine, pentobarbital-d5, and prazepam are used as internal standards.

## 2 SCOPE

2.1 This procedure functions as an initial screen for multiple drugs of interest from toxicology specimens, including but not limited to blood and urine. Urine specimens are always reported qualitatively.

2.2 This procedure may be suitable for the analysis of benzodiazepines, barbiturates, anticonvulsants, antihistamines, tricyclic antidepressants, non-tricyclic antidepressants (such as selective serotonin reuptake inhibitors (SSRIs) and related compounds), muscle relaxants, narcotic analgesics, dissociative anesthetics, and other abused drugs. Other targeted procedures are available for several basic, acidic, and neutral drugs. For samples containing benzodiazepines, Alprazolam Confirmation and Benzodiazepines Confirmation are available.

2.3 Targeted methods of analysis with greater sensitivity are available for many commonly encountered drugs. Thirty drugs were selected to represent the wide range of drugs and drug classes that are commonly detected using this procedure.

## 3 STANDARDS AND SOLUTIONS

3.1 BAN Working Mix

The BAN Working Standard is a methanolic solution of common basic, acidic, and neutral (BAN) drugs. Refer to the Reagent QC Log for composition of the current BAN mix. Typical drugs in the BAN working standard include benzodiazepines, barbiturates, muscle relaxants, opioids, tricyclic antidepressants, selective serotonin reuptake inhibitors or selective norepinephrine reuptake inhibitors, dissociative anesthetics and other abused drugs. For example: butalbital, secobarbital, phenobarbital, benzylpiperazine, meperidine, meprobamate, ketamine, diphenhydramine, carisoprodol, PCP, tramadol, methadone, propoxyphene, amitriptyline, nortriptyline, imipramine, cyclobenzaprine, hydrocodone, oxycodone, zolpidem, diazepam, nordiazepam, flurazepam, alprazolam, venlafaxine, and sertraline.

3.1.1 Preparation of BAN Working Mix (20 ng/µL):

Separate calibrator and quality control working mixes are prepared at 20 ng/ml. Drug standards are purchased in 1.0 mg/mL ampoules. In a 10 mL volumetric flask, add 200 µL of undiluted drug standard (1.0 mg/mL). Bring to volume with methanol. Store refrigerated (3 month expiration). Refer to fortification guide below for guidance on preparation of calibrators and controls.

3.1.2 Preparation of BAN Working Mix (2 ng/µL):

In a 10 mL volumetric flask, add 1.0 mL of BAN Working Mix (20 ng/µL). Bring to volume with methanol. Store refrigerated (3 month expiration). Refer to fortification guide below for guidance on preparation of calibrators and controls.

3.2 BAN Internal Standard Solution:

This solution consists of mepivacaine (0.01 mg/mL), prazepam (0.01 mg/mL), and pentobarbital–$D_5$ (0.01 mg/mL).

Preparation of BAN Internal Standard Solution:

In a 10 mL volumetric flask, add 1.0 mL mepivacaine (0.1 mg/mL), 1.0 mL prazepam (0.1 mg/mL) and 1.0 mL of pentobarbital-$D_5$ (0.1 mg/mL), and bring to volume with methanol. Store refrigerated (6 month expiration).

3.3 1M Acetic Acid
3.4 100 mM, pH 6.0 Phosphate buffer
3.5 Methylene Chloride/Isopropanol (80:20)
3.6 Elution Solvent: 2% ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol
3.7 Acidic Methanol (1 % HCl in methanol)

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)
4.2 Cerex PolyChrom Clin II SPE columns (Black ones)
4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold
4.4 Pan balance
4.5 pH meter
4.6 Evaporator
4.7 Vortex mixer
4.8 Centrifuge
4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.2 mL/min with an injection volume of 2 µL in split mode (10:1).
5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 150 °C<br>Hold for 0.5 minutes |

| | |
|---|---|
| | 20 °C/min to 180 °C<br>Hold for 2 minutes<br>5 °C/min to 230 °C<br>Hold for 1 minute<br>10 °C/min to 250 °C<br>Hold for 0.5 min<br>30 °C/min to 290 °C<br>Hold for 6 min |
| Total Run Time: | 24.833 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

**BLOOD, URINE AND ALL OTHER MATRICES**

6.1 In a round bottom glass culture or centrifuge tube, add 2 mL of blood and 100 µL Internal Standard Solution while vortex mixing. NOTE 1: IT IS RECOMMENDED TO CENTRIFUGE SPECIMENS IN PREPARATION FOR EXTRACTION. NOTE 2: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME, ADD BUFFER AS THE DILUENT AND DOCUMENT IN THE CASE RECORD.

6.2 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. A minimum of one in-house control should be included in each run. Use appropriate external controls (e.g. UTAK Whole Blood Controls) when available.

6.3 Add 4 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex. Centrifuge for at least 10 minutes at 4000 rpm.

6.4 Place collection tubes or reservoir (for waste) into vacuum manifold rack/positive pressure apparatus.

6.5 Pour sample onto SPE columns. Use sufficient vacuum/pressure to draw samples through the columns.

6.6 Wash column with 1 mL deionized water.

6.7 Remove waste tubes/reservoir and replace with new.

6.8 Wash column with 1 mL of 1 M acetic acid.

6.9 Dry columns for 5 minutes (at full vacuum).

6.10 Wash column with 1 mL hexane.

6.11 Place conical collection tubes into the vacuum manifold/positive pressure apparatus. If the Teflon inserts appear dirty (vacuum manifold), replace them.

6.12 Elute acidic and neutral drugs with 1 mL ethyl acetate.
6.13 Turn on vacuum for a few seconds to ensure all elution solvent has drained from the column.
6.14 Remove conical collection tubes and replace with waste tubes/reservoir.
6.15 Wash column with 1 mL methanol.
6.16 Dry column for 5 minutes (at full vacuum) or pressure as appropriate.
6.17 Replace the conical collection tubes containing the acidic/neutral fraction into the vacuum manifold/positive pressure apparatus. If the Teflon inserts appear dirty, replace them.
6.18 Elute basic drugs with 1 mL elution solvent (2% ammonium hydroxide in methylene chloride/isopropyl alcohol 80:20). ELUTION SOLVENT MUST BE MADE FRESH.
6.19 Turn on vacuum or apply pressure for a few seconds to ensure all elution solvent has drained from the column.
6.20 Remove tubes from rack and add 30 µL of acidic methanol.
6.21 Evaporate to dryness under nitrogen for 15 minutes at 50-60°C. Reconstitute in 30 µL ethyl acetate (or an appropriate volume), vortex, and transfer to autosampler vials.
6.22 Inject 2 µL onto the GC/MS using BAN.M.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.
7.2 FULL-SCAN acquisition: BAN.M

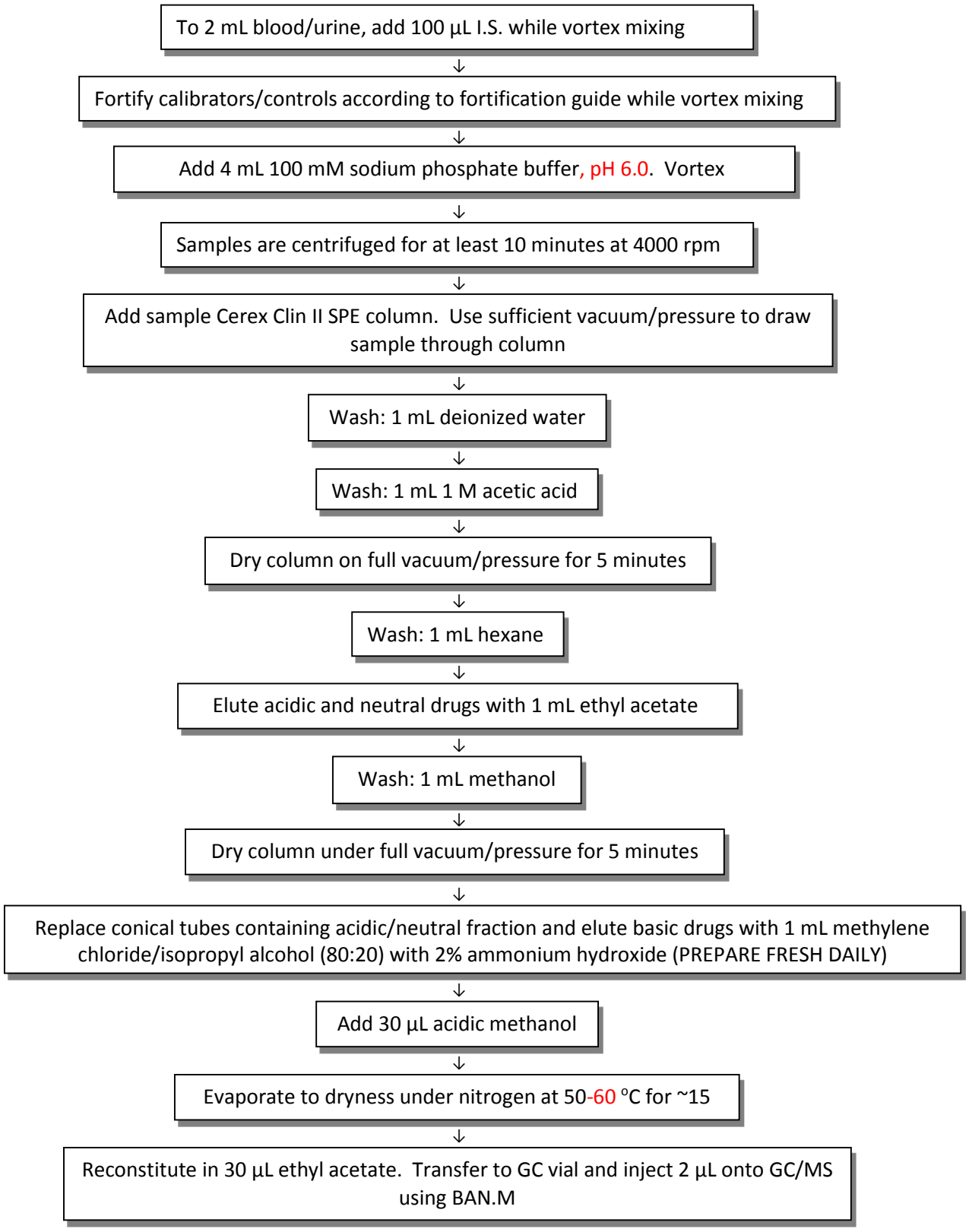
## 8 INTERPRETATION OF RESULTS

8.1 Qualitative identification is determined using relative retention time (RRT) and mass spectral characteristics. Relative retention times must be within 2% of the standard or QC and acceptable mass spectral library fit. See “Current Working RRT List” for RRTs of common drugs.
8.2 Quantitative results are determined using drug standards of known concentration. Quantitative analyses are not performed on urine samples. For blood samples, drug-free blood is fortified with the drugs of interest. Limits of detection for selected basic, acidic, and neutral drugs can be found in the respective procedure’s validation.

## 9 QUANTITATIVE ANALYSIS

9.1 Specific (targeted) quantitation procedures utilizing selected ion monitoring (SIM) and deuterated internal standards are available for many commonly encountered drugs.
9.2 Mepivacaine, pentobarbital-d5, and prazepam are used as internal standards for full scan acquisition. Other internal standards can be used for the quantitation of other basic, acidic or neutral drugs using either full scan or SIM analysis. Deuterated internal standards are preferred, if available. Refer to the drug inventory for a current list of deuterated internal standards.
9.3 If selected ion monitoring (SIM) is used, refer to the appropriate Method File for the GC/MS. For SIM, all three characteristic ions must be present. Ion intensity and ratios should be taken into consideration. Ion ratios should be within 20% of the appropriate calibrator or QC. If ion ratios are out of range, quantitative data may be reported if the ratios are within ± 20% of the nearest calibrator or QC. However, ions of low intensity may not be within this range for all case samples. These are evaluated on a case by case basis and are subject to technical and administrative review.

## 10 FLOWCHART FOR BASIC, ACIDIC, AND NEUTRAL DRUG ANALYSIS


To 2 mL blood/urine, add 100 µL I.S. while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 4 mL 100 mM sodium phosphate buffer, pH 6.0. Vortex
Samples are centrifuged for at least 10 minutes at 4000 rpm
Add sample Cerex Clin II SPE column. Use sufficient vacuum/pressure to draw sample through column
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes
Wash: 1 mL hexane
Elute acidic and neutral drugs with 1 mL ethyl acetate
Wash: 1 mL methanol
Dry column under full vacuum/pressure for 5 minutes
Replace conical tubes containing acidic/neutral fraction and elute basic drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH DAILY)
Add 30 µL acidic methanol
Evaporate to dryness under nitrogen at 50-60 °C for ~15
Reconstitute in 30 µL ethyl acetate. Transfer to GC vial and inject 2 µL onto GC/MS using BAN.M

## 11 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | BAN Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 2 | 40 | 2 | 40 |
| 2 | 60 | 2 | 60 |
| 2 | 100 | 2 | 100 |
| 2 | 250 | 20 | 25 |
| 2 | 500 | 20 | 50 |
| 2 | 1000 | 20 | 100 |

## 12 LITERATURE AND SUPPORTING DOCUMENTATION

12.1 Chen, Xiao-Hua, et al. Isolation of Acidic, Neutral, and Basic Drugs from Whole Blood Using A Single Mixed-Mode Solid-Phase Extraction Column. J Anal Toxicol 1992 Nov-Dec; Vol. 16 (6), pp. 351-355.

12.2 Method File: BAN.M

# Benzodiazepine Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PRINCIPLE

A targeted analysis is performed for desalkylflurazepam, flurazepam, nordiazepam, oxazepam, diazepam, lorazepam, temazepam, clonazepam, and alpha-hydroxyalprazolam. Drugs are isolated from the matrix using liquid-liquid extraction (LLE). Trimethylsilyl derivatives of each drug and deuterated internal standards are prepared and analyzed using GC/MS, selective ion monitoring (SIM), and electron (EI) ionization.

## 2 SCOPE

Confirmatory analysis of target analytes from toxicology specimens including but not limited to blood and urine. Urine confirmations are reported only qualitatively.

## 3 REAGENTS AND STANDARDS

3.1 BENZO Working Standards:

Two working standards are routinely used. Each contains desalkylflurazepam, flurazepam, nordiazepam, oxazepam, diazepam, lorazepam, temazepam, clonazepam, and alpha-hydroxyalprazolam at either 10 ng/µL or 1 ng/µL. Additional working standards may be prepared as needed.

3.1.1 Preparation of 10 ng/µL BENZO Working Standard: Add 1000 µL of each drug standard (0.1 mg/mL) to a 10mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.1.2 Preparation of 1 ng/µL Working Standard: Add 1000 µL of the 10 ng/µL BENZO Working Standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (1 month expiration).

3.2 Internal Standard Solution: This solution consists of desalkylflurazepam-d4, nordiazepam-d5, oxazepam-d5, diazepam-d5, lorazepam-d4, temazepam-d5, clonazepam-d4, and alph-hydroxyalprazolam-d5 (2 ng/µL) in methanol.

Preparation of (2 ng/µL) Internal Standard Solution: Add 200 µL of each deuterated drug standard (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.3 KCl Stock Solution, 200 mg/mL: Add 20 g KCl to a 100 mL volumetric flask. Bring to volume with deionized water. Store at room temperature (12 month expiration).

3.4 NaOH Stock Solution, 100 mg/mL: Add 10 g NaOH to a 100 mL volumetric flask. Bring to volume with deionized water. Store at room temperature (12 month expiration).

3.5 KCl/NaOH Working Solution, pH 12: Add 4.8 mL NaOH Stock Solution and 18.6 mL KCl Stock Solution to a 1 L volumetric flask. Bring to volume with deionized water. Check pH to ensure 12. Adjust pH as needed. Store at room temperature (12 month expiration).

3.6 1:10 KCl/NaOH Working Solution, pH 12: Add 100 mL KCl/NaOH Working Solution to a 1 L volumetric flask. Bring to volume with deionized water. Store at room temperature (12 month expiration).

3.7 1-Chlorobutane
3.8 BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) for derivatization. Must be refrigerated.
3.9 Deconjugation Enzyme: E-Coli Type IX-A β-Glucuronidase (125,000 Units). To one vial of 125,000 Units, add 25 mL of deconjugation buffer and mix gently. Store frozen aliquots to prevent freeze-thaw cycles. Expiration of reconstituted enzyme reagent is 30 days. Do not re-freeze once reconstituted.
3.10 Deconjugation Buffer (100 mM sodium phosphate buffer, pH 6.8): Add 7.0 g of dibasic sodium phosphate to a 500 mL volumetric flask and bring to volume with deionized water while stirring. In a second 500 mL volumetric flask, add 6.0 g of monobasic sodium phosphate and bring to volume with deionized water while stirring. Mix the two solutions together until a pH of 6.8 is obtained. Refrigerated storage is recommended (expiration 6 months).
3.11 Conjugated Oxazepam Working Standard (10 ng/µL): An oxazepam-glucuronide working standard is used. Add 100 µL of 0.1 mg/mL oxazepam-glucuronide drug standard to a 10 mL volumetric flask. Bring to volume with methanol. Store refrigerated (6 month expiration). Other conjugated benzodiazepines may be used (e.g. lorazepam-glucuronide) if oxazepam-glucuronide is not available.

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)
4.2 Tube rotator
4.3 Pan balance
4.4 pH meter
4.5 Evaporator
4.6 Vortex mixer
4.7 Centrifuge
4.8 Tissue homogenizer
4.9 Sonicator
4.10 Heating block
4.11 Vacuum pump or house vacuum
4.12 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.5 mL/min with an injection volume of 2 µL in splitless mode.

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 180 °C<br>Hold for 0.25 minutes<br>15 °C/min to 240 °C<br>Hold for 3 minutes<br>30 °C/min to 290 °C<br>Hold for 5 minutes |
| Total Run Time: | 13.917 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE:

**BLOOD:**

6.1.1 In a round bottom glass centrifuge tube, add 1 mL of blood. NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME AND DOCUMENT IN THE CASE FOLDER. For qualitative analysis include a minimum of one negative and one positive control in each run. For preparation of a 100 ng/mL positive control, add 100 µL of BENZO Working Standard Solution (1 ng/µL) to 1 mL blood while vortex mixing.

6.1.2 Add 50 µL of Internal Standard (2 ng/µL) while vortex mixing. Final concentration of internal standard in each sample is 100 ng/mL.

6.1.3 Fortify all controls and calibrators while vortex mixing using appropriate amount of working standard. Refer to the fortification guide for preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. A minimum of one in-house control should be included in each run. Use appropriate external controls (eg: Utak Whole Blood Controls) when available.

6.1.4 Add 1 mL of 100 mM pH 6.8 phosphate buffer (deconjugation buffer) and vortex.

6.1.5 Add 2 mL KCl/NaOH Working Solution to each tube. Vortex.

6.1.6 Add 6 mL n-butyl chloride to each tube. Cap tightly and mix on tube rotator for 5 minutes.

6.1.7 Centrifuge for 5 minutes at 4000 rpm.

6.1.8 Transfer the organic (upper) layer into a clean round bottom centrifuge tube.

6.1.9 Add 1 mL 1:10 KCl/NaOH, pH 12. NOTE: DO NOT VORTEX.

6.1.10 Rotate tubes for 5 minutes.

6.1.11 Centrifuge for 5 minutes at 4000 rpm.
6.1.12 Transfer the organic (upper) layer into a clean conical bottom glass tube.
6.1.13 Evaporate to dryness under nitrogen for 10 minutes at 50 °C.
6.1.14 IN THE FUME HOOD add 10 µL ethyl acetate and 25 µL BSTFA, vortex, and cap tightly. Heat at 70 °C for 15 minutes.
6.1.15 Remove from heat block and cool to room temperature.
6.1.16 Transfer to autosampler vials. Reconstitution volumes may be adjusted with ethyl acetate prior to injection when necessary.
6.1.17 Inject 2 µL onto the GC/MS using BENZOSIM.M.

6.2 **BLOOD (DECONJUGATION)-OPTIONAL (This procedure is recommended when the EIA screen results for Benzodiazepines is high but confirmation by GCMS is negative)**

If deconjugation of glucuronidated benzodiazepines in blood is required, proceed as follows:

6.2.1 In a round bottom glass centrifuge tube, add 1 mL of sample. NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME AND DOCUMENT IN THE CASE FOLDER. For qualitative analysis include a minimum of one negative and one positive control in each run. For preparation of a 100 ng/mL positive control, add 100 µL of BENZO Working Standard Solution (1 ng/µL) to 1 mL blood while vortex mixing. For hydrolyzation of glucuronidated drugs, a glucuronidated control must be included. For preparation of a 200 ng/mL oxazepam-glucuronide control, add 20 µL of oxazepam-glucuronide (10 ng/µL) to 1 mL blood while vortex mixing. Other glucuronidated drugs may be used when available.
6.2.2 Add 1 mL of deconjugation buffer and vortex.
6.2.3 Add 100 µL deconjugation enzyme and vortex.
6.2.4 Cap and heat samples at 37 °C for 1 hour. Let cool to room temperature, then proceed as outlined blow.
6.2.5 Add 50 µL Internal Standard Solution (2 ng/µL) while vortex mixing. The final concentration of internal standard is 100 ng/mL.
6.2.6 Proceed from step 4 of blood procedure above.

6.3 **URINE (ALL SPECIMENS ARE DECONJUGATED)**
6.3.1 In a round bottom glass centrifuge tube, add 1 mL of sample. NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME AND DOCUMENT IN THE CASE FOLDER. For qualitative analysis include a minimum of one negative and one positive control in each run. For preparation of a 100 ng/mL positive control, add 100 µL of BENZO Working Standard Solution (1 ng/µL) to 1 mL urine while vortex mixing. For hydrolyzation of glucuronidated drugs, a glucuronidated control must be included. For preparation of a 200 ng/mL oxazepam-glucuronide control, add 20 µL of oxazepam-glucuronide (10 ng/µL) to 1 mL urine while vortex mixing. Other glucuronidated drugs may be used when available.
6.3.2 Add 1 mL of deconjugation buffer to all tubes and vortex.

6.3.3 Add 100 µL deconjugation enzyme to all tubes and vortex.

6.3.4 Cap and heat samples at 37°C for 1 hour. Let cool to room temperature, then proceed as outlined blow.

6.3.5 Add 100 µL Internal Standard Solution (2 ng/µL) while vortex mixing. The final concentration of internal standard is 200 ng/mL.

6.3.6 Proceed from step 4 of blood procedure above.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.

7.2 SIM acquisition: BENZOSIM.M.

| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Desalkylflurazepam | 360 | 325, 245 | 6.40 |
| Flurazepam | 86 | 387, 315 | 10.35 |
| Nordiazepam | 341 | 327, 227 | 6.55 |
| Oxazepam | 429 | 401, 313 | 7.61 |
| Diazepam | 283 | 256, 221 | 8.29 |
| Lorazepam | 429 | 449, 347 | 8.48 |
| Temazepam | 343 | 372, 283 | 9.19 |
| Clonazepam | 387 | 372, 352 | 9.55 |
| α-Hydroxyalprazolam | 396 | 383, 364 | 12.55 |
| **Internal Standards** | **Quant Ion** | **Qualifier Ions** | **RT*** |
| Desalkylflurazepam-D4** | 364 | 329, 249 | 6.36 |
| Nordiazepam-d5 | 347 | 332, 232 | 6.50 |
| Oxazepam-d5 | 433 | 406, 318 | 7.57 |
| Diazepam-d5 | 289 | 261, 226 | 8.28 |
| Lorazepam-d4 | 433 | 453, 351 | 8.46 |
| Temazepam-d5 | 348 | 377, 288 | 9.20 |
| Clonazepam-d4 | 391 | 376, 356 | 9.54 |
| α-Hydroxyalprazolam-d5 | 401 | 388, 386 | 12.52 |

*Retention Time (RT) varies with column length. Deuterated (D4 and D5) internal standards are used throughout. Refer to attached method for acquisition parameters. **Desalkylflurazepam-d4 is used for both desalkylflurazepam and flurazepam.

## 8 INTERPRETATION OF RESULTS

8.1 Assay performance including but not limited to precision, accuracy, limits of detection, quantitation, and linearity are summarized in the validation documentation.

8.2 The limit of detection in blood and urine are as follows:

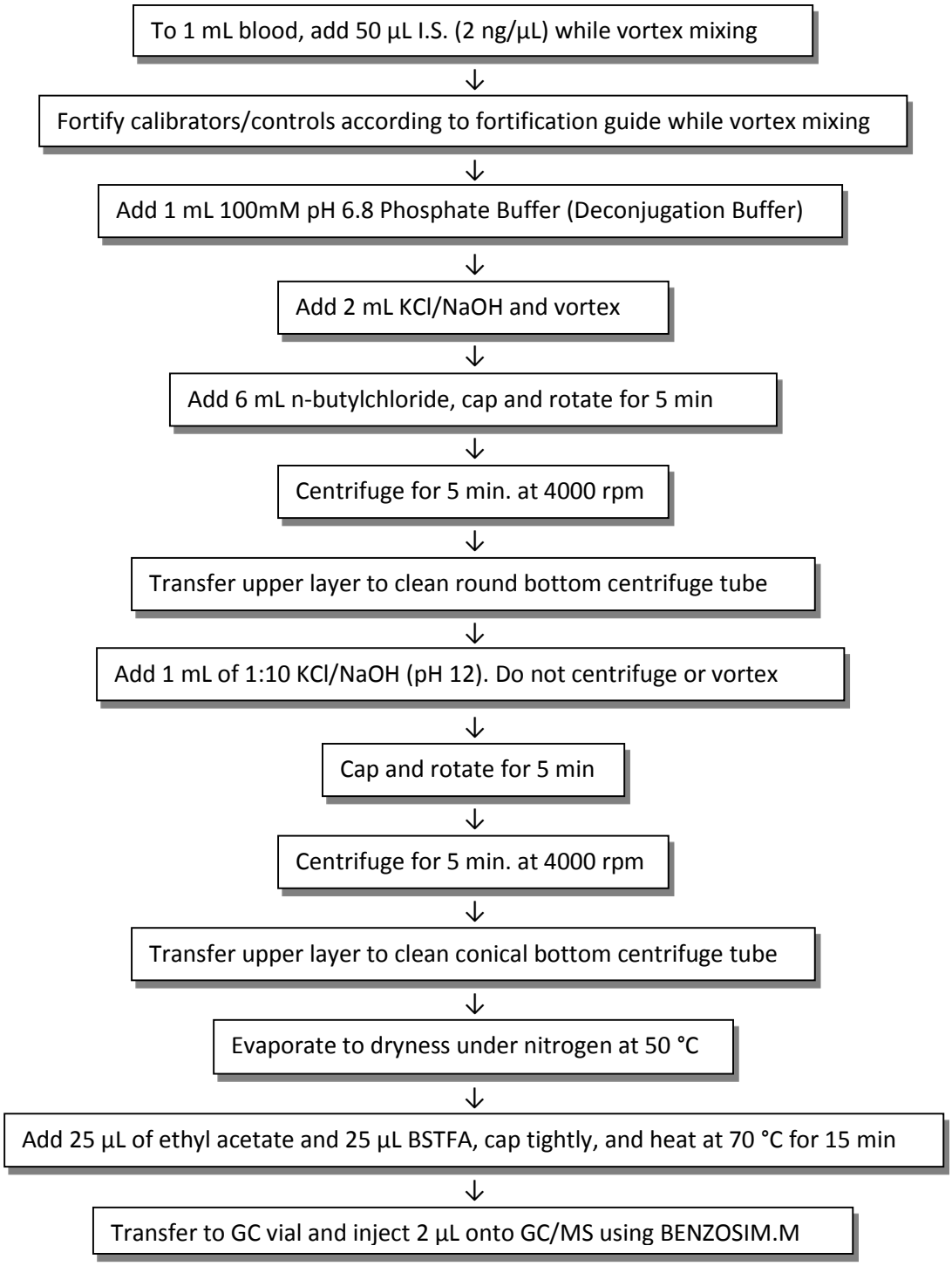
| Drug | LOD and LOQ (Blood) ng/mL | LOD (Urine) ng/mL |
|---|---|---|
| Desalkylflurazepam | 15 | 15 |
| Flurazepam | 10 | 10 |
| Nordiazepam | 15 | 20 |
| Oxazepam | 15 | 15 |
| Diazepam | 25 | 15 |
| Lorazepam | 25 | 20 |
| Temazepam | 10 | 10 |
| Clonazepam | 30 | 10 |
| α-Hydroxyalprazolam | 30 | 20 |

8.3 Quality Controls

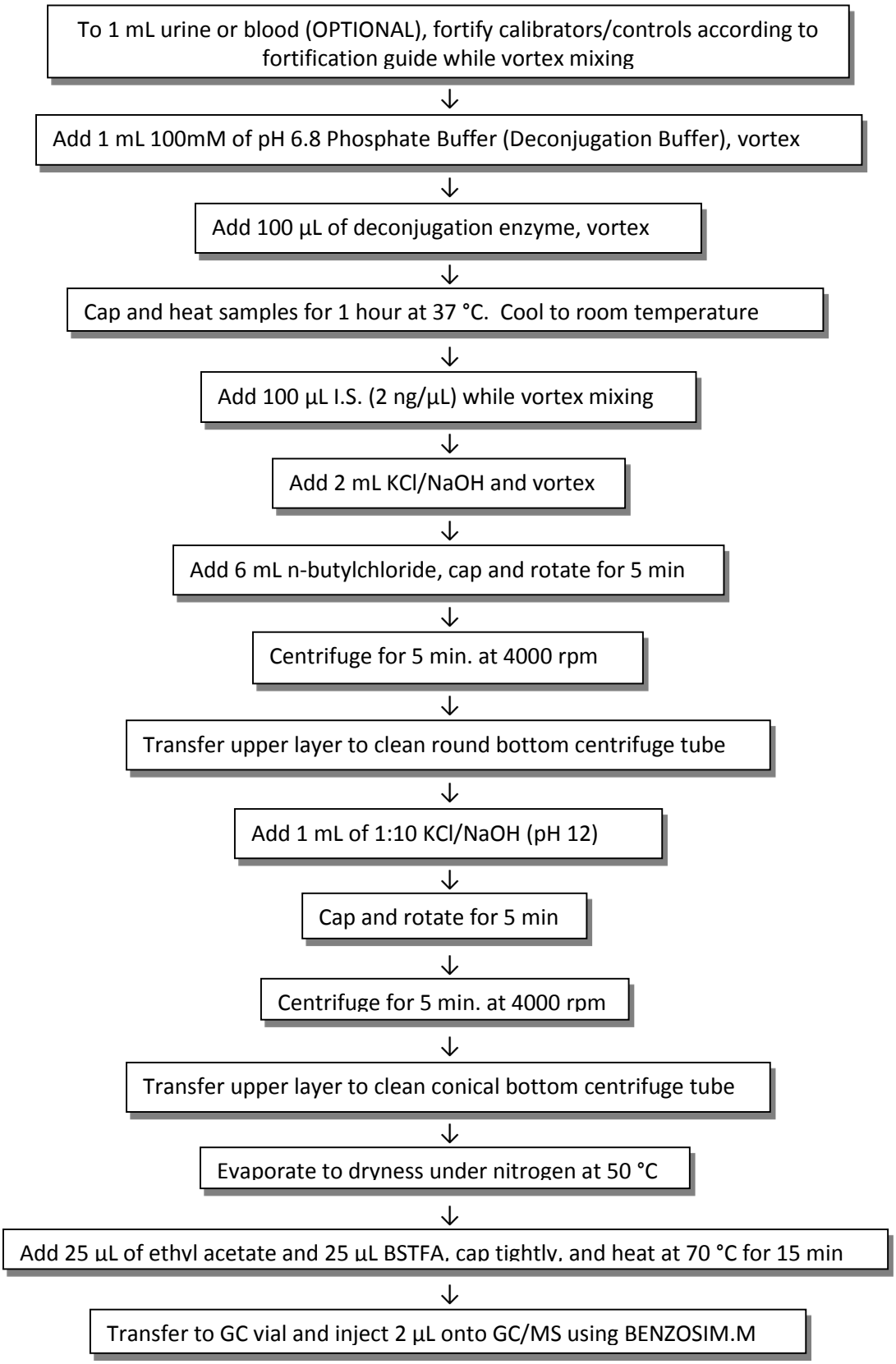
For quantitative analysis, use external QCs (e.g. UTAK QCs) when available. UTAK "Benzodiazepines Plus" 100 ng/mL QC contains 100 ng/mL of flurazepam, nordiazepam, oxazepam, diazepam, lorazepam, temazepam, clonazepam*****, and alpha-hydroxyalprazolam. Record the calculated concentrations in the appropriate QC Log. NOTE: Although present in the UTAK QC, clonazepam cannot be quantitated due to an interference caused by prazepam (also in the UTAK QC) with the target ion.

Qualitative identification is determined using characteristic retention times and mass spectral characteristics. Ion ratios should be within +/- 20% of the calibrator or QC. However, ions of low intensity (<10%) may not be within this range and are acceptable if they fall within 4% (absolute) of the established ion ratio. These are evaluated on a case by case basis.

## 9 FLOWCHART FOR BLOOD BENZODIAZEPINE ANALYSIS (NO DECONJUGATION)


To 1 mL blood, add 50 µL I.S. (2 ng/µL) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 1 mL 100mM pH 6.8 Phosphate Buffer (Deconjugation Buffer)
Add 2 mL KCl/NaOH and vortex
Add 6 mL n-butylchloride, cap and rotate for 5 min
Centrifuge for 5 min. at 4000 rpm
Transfer upper layer to clean round bottom centrifuge tube
Add 1 mL of 1:10 KCl/NaOH (pH 12). Do not centrifuge or vortex
Cap and rotate for 5 min
Centrifuge for 5 min. at 4000 rpm
Transfer upper layer to clean conical bottom centrifuge tube
Evaporate to dryness under nitrogen at 50 °C
Add 25 µL of ethyl acetate and 25 µL BSTFA, cap tightly, and heat at 70 °C for 15 min
Transfer to GC vial and inject 2 µL onto GC/MS using BENZOSIM.M

## 10 FLOWCHART FOR DECONJUGATED BENZODIAZEPINE ANALYSIS


To 1 mL urine or blood (OPTIONAL), fortify calibrators/controls according to fortification guide while vortex mixing
Add 1 mL 100mM of pH 6.8 Phosphate Buffer (Deconjugation Buffer), vortex
Add 100 µL of deconjugation enzyme, vortex
Cap and heat samples for 1 hour at 37 °C. Cool to room temperature
Add 100 µL I.S. (2 ng/µL) while vortex mixing
Add 2 mL KCl/NaOH and vortex
Add 6 mL n-butylchloride, cap and rotate for 5 min
Centrifuge for 5 min. at 4000 rpm
Transfer upper layer to clean round bottom centrifuge tube
Add 1 mL of 1:10 KCl/NaOH (pH 12)
Cap and rotate for 5 min
Centrifuge for 5 min. at 4000 rpm
Transfer upper layer to clean conical bottom centrifuge tube
Evaporate to dryness under nitrogen at 50 °C
Add 25 µL of ethyl acetate and 25 µL BSTFA, cap tightly, and heat at 70 °C for 15 min
Transfer to GC vial and inject 2 µL onto GC/MS using BENZOSIM.M

## 11 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 25 | 1 | 25 |
| 1 | 50 | 1 | 50 |
| 1 | 100 | 1 | 100 |
| 1 | 250 | 10 | 25 |
| 1 | 500 | 10 | 50 |
| 1 | 1000 | 10 | 100 |

## 12 LITERATURE AND SUPPORTING DOCUMENTATION

12.1 Gunnar, T; Ariniemi, K; Lillsunde, P. Determination of 14 benzodiazepines and hydroxyl metabolites, zaleplon and zolpidem as tert-butyldimethylsilyl derivatives compared with other common silylating reagents in whole blood by gas chromatography-mass spectrometry. J Chrom B 2005 Nov-Dec; Vol. 818 (2), pp. 175-189.

12.2 Jufer-Phipps, RA; Levine, B. Benzodiazepines in Principles of Forensic Toxicology, 3rd Edition. Levine B (Ed). AACC Press, Washington DC, 2009, pp 191-205.

12.3 Siek, TJ. Specimen Preparation: Liquid-Liquid Extraction in Principles of Forensic Toxicology, 3rd Edition. Levine B (Ed). AACC Press, Washington DC, 2009, pp71-77.

12.4 Tiscione, NB, Shan, X; Alford, I; Yeatman, DT. Quantitation of Benzodiazepines and in Whole Blood by Electron Impact-Gas Chromatography-Mass Spectrometry. J Anal Toxicol 2008 Nov-Dec; Vol. 32 (8), pp. 644-52.

12.5 Method file: BENZOSIM.M.

# Cannabinoid Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for Δ9-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THCA). Drugs are isolated from the matrix using solid phase extraction (SPE). Trimethylsilyl derivatives are prepared and analyzed using gas chromatography/mass spectrometry (GC/MS), deuterated internal standards, and selective ion monitoring (SIM) in electron ionization (EI) mode.

## 2 SCOPE

Confirmatory analysis of target analytes from toxicology specimens including but not limited to blood and urine. Urine confirmations are reported only qualitatively.

## 3 STANDARDS AND SOLUTIONS

3.1 THC/THCA Working Standards

Three working standards are used. Each contains THC and THCA at either 10, 1, or 0.1 ng/µL, respectively.

3.1.1 Preparation of 10 ng/µL THC/THCA Working Standard: Add 1000 µL of THC and THCA (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.1.2 Preparation of 1 ng/µL Working Standard: Add 1000 µL of the 10 ng/µL THC/THCA Working Standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.1.3 Preparation of 0.1 ng/µL Working Standard: Add 1000 µL of the 1 ng/µL THC/THCA Working Standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.1.4 Preparation of 10 ng/µL THCA-Glucuronide Stock Standard: Add 1000 µL of THCA-Glucuronide (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.1.5 Preparation of 1 ng/µL THCA-Glucuronide Working Standard: Add 1000 µL of THCA-Glucuronide (10 ng/µL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2 Internal Standard Solution – This solution consists of THC-d3 and THCA-d3 in methanol at 1 ng/µL.

3.2.1 Preparation of (10 ng/µL) Internal Standard Stock Solution: Add 1000 µL of each deuterated drug standard (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (12 month expiration).

3.2.2 Preparation of (1 ng/µL) Internal Standard Solution: Add 1000 µL of 10 ng/µL internal standard stock solution to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 months expiration).

3.3 Cold Acetonitrile: HPLC grade acetonitrile used in this procedure can be stored in the refrigerator or freezer prior to use.

3.4 10 M KOH: Add 56.10 g KOH pellets to a 100 milliliter volumetric flask. Bring to volume with deionized water. Stir. Let cool and check volume as exothermic reaction may have an effect on volume. Store at room temperature (12 month expiration).

3.5 $H_2O$ / Acetonitrile (85:15): In a 1 liter volumetric flask, add 150 mL acetonitrile. Fill to volume with deionized water. Store at room temperature (expiration 6 months).

3.5.1 SPE Wash Solution: 1% ammonium hydroxide (concentrated) in 85:15 $H_2O$ / Acetonitrile. For example: In a 25 mL volumetric flask, add 0.25 mL concentrated ammonium hydroxide. Bring to volume with 85:15 $H_2O$ / Acetonitrile. MUST BE PREPARED FRESH.

3.5.2 Hexane / Ethyl Acetate (90:10): In a 1 liter volumetric flask, add 100 mL of ethyl acetate. Bring to volume with hexane. Store at room temperature (expiration 6 months).

3.5.3 Elution Solvent: 3% glacial acetic acid (concentrated) in 90:10 hexane/ethyl acetate. For example: In a 50 mL volumetric flask, add 1.5 mL glacial acetic acid. Bring to volume with 90:10 hexane/ethyl acetate. MUST BE PREPARED FRESH.

3.5.4 BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) for derivatization. Must be refrigerated.

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)
4.2 Cerex PolyChrom Clin II SPE columns
4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold
4.4 Pan balance
4.5 pH meter
4.6 Evaporator
4.7 Vortex mixer
4.8 Centrifuge
4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 0.8 mL/min with an injection volume of 2 µL in splitless mode.

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 160 °C<br>Hold for 0.5 minutes<br>40 °C/min to 230 °C<br>Hold for 6 minutes<br>30 °C/min to 290 °C<br>Hold for 8 minutes |
| Total Run Time: | 18.25 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

**BLOOD (USE SALINIZED GLASSWARE)**

6.1.1 In a round bottom glass centrifuge tube add 1 mL blood and 50 µL Internal Standard Solution (1 ng/µL) while vortex mixing. Final concentration of internal standard in each sample of 50 ng/mL. NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME, AND DOCUMENT IN THE CASE RECORD.

6.1.2 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. A minimum of one in-house control (e.g., 20 ng/mL THC/THCA) should be included in each run. Use appropriate external controls (e.g. UTAK Whole Blood Controls) when available.

6.1.3 Precipitate proteins by addition of 2 mL cold acetonitrile while vortex mixing.

6.1.4 Centrifuge for 10 minutes at 4000 rpm.

6.1.5 Pour off the supernatant layer into a clean round bottom glass tube. Add 2 mL of deionized water.

6.1.6 Place collection tubes or reservoir (for waste) into vacuum manifold rack or on positive pressure apparatus.

6.1.7 Pour sample onto SPE columns. Samples should flow under gravity. Do not use the vacuum (or pressure) unless it is necessary.

6.1.8 Wash column with 1 mL SPE wash solution. (1% ammonium hydroxide (concentrated) in 85:15 $H_2O$/Acetonitrile.) WASH SOLUTION MUST BE MADE FRESH.

6.1.9 Dry column on full vacuum/pressure for 5 minutes.

6.1.10 Place conical glass collection tubes into the rack.

6.1.11 Elute THC with 2 mL ethyl acetate.

6.1.12 Dry column on full vacuum/pressure for 5 minutes. .

6.1.13 Elute THCA with 2 mL SPE Elution solvent (3% glacial acetic acid in hexane/ethyl acetate 90:10). ELUTION SOLVENT MUST BE MADE FRESH.

6.1.14 Turn on vacuum (or apply pressure) for a few seconds to ensure all elution solvent has drained from the column.

6.1.15 Remove tubes from rack and evaporate to dryness (approximately 20 minutes) under nitrogen at 50-60 °C.

6.1.16 IN THE FUME HOOD, add 30 µL BSTFA. Cap tightly. Heat at 70 °C for 15 minutes.

6.1.17 Allow samples to cool to room temperature. Transfer to auto-sampler vials.

6.1.18 Inject 2 µL onto the GC/MS using THC.M. Note: Ethyl acetate (HPLC grade) may be added to samples that require dilution (i.e. overloaded peaks), if necessary.

6.2 **URINE ( USE SALINIZED GLASSWARE)**

6.2.1 In a round bottom centrifuge tube, add 1 mL urine
For qualitative analysis include appropriate negative and positive controls. For preparation of a 50 ng/mL positive control, add 50 µL of THC working Standard Solution (1 ng/µL) to 1 mL urine while vortex mixing. A glucuronidated control should also be included. For preparation of a 50 ng/mL THC-glucuronide control, add 50 µL of THCA-glucuronide (1ng/µL) to 1 mL urine while vortex mixing.

6.2.2 Add 100 µL of 10 M KOH and vortex.

6.2.3 Heat samples at 60 °C for 15 minutes. Let cool to room temperature.

6.2.4 Add 100 µL Internal Standard Solution (1 ng/µL) while vortex mixing. The total concentration of internal standard is 100 ng/mL.

6.2.5 If sample is cloudy or contains particulates, centrifuge for 10 minutes at 4000 rpm.

6.2.6 Proceed from step 6 through 18 of blood procedure. It is acceptable to increase the reconstitution volume for urine extracts prior to injection to prevent sample overload. Ethyl acetate may be used for sample dilution or reconstitution of derivatized extracts. The presumptive screening test results can be used as a guide.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.

| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| THC | 386 | 371, 303 | 6.67 |
| THC-d3 | 389 | 374, 306 | - |
| THCA | 473 | 371, 488 | 12.49 |
| THCA-d3 | 476 | 374, 491 | - |

*Retention Time (RT) varies with column length. Deuterated (D3) internal standards are used throughout. Corresponding ions for internal standards are M+3. Refer to attached method for acquisition parameters (THC.M)

7.2 SIM acquisition: THC.M. Use this method to perform targeted analysis for THC and THCA.

## 8 INTERPRETATION OF RESULTS

8.1 Assay performance including but not limited to precision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.

8.2 Limits of detection in blood and urine are as follows:

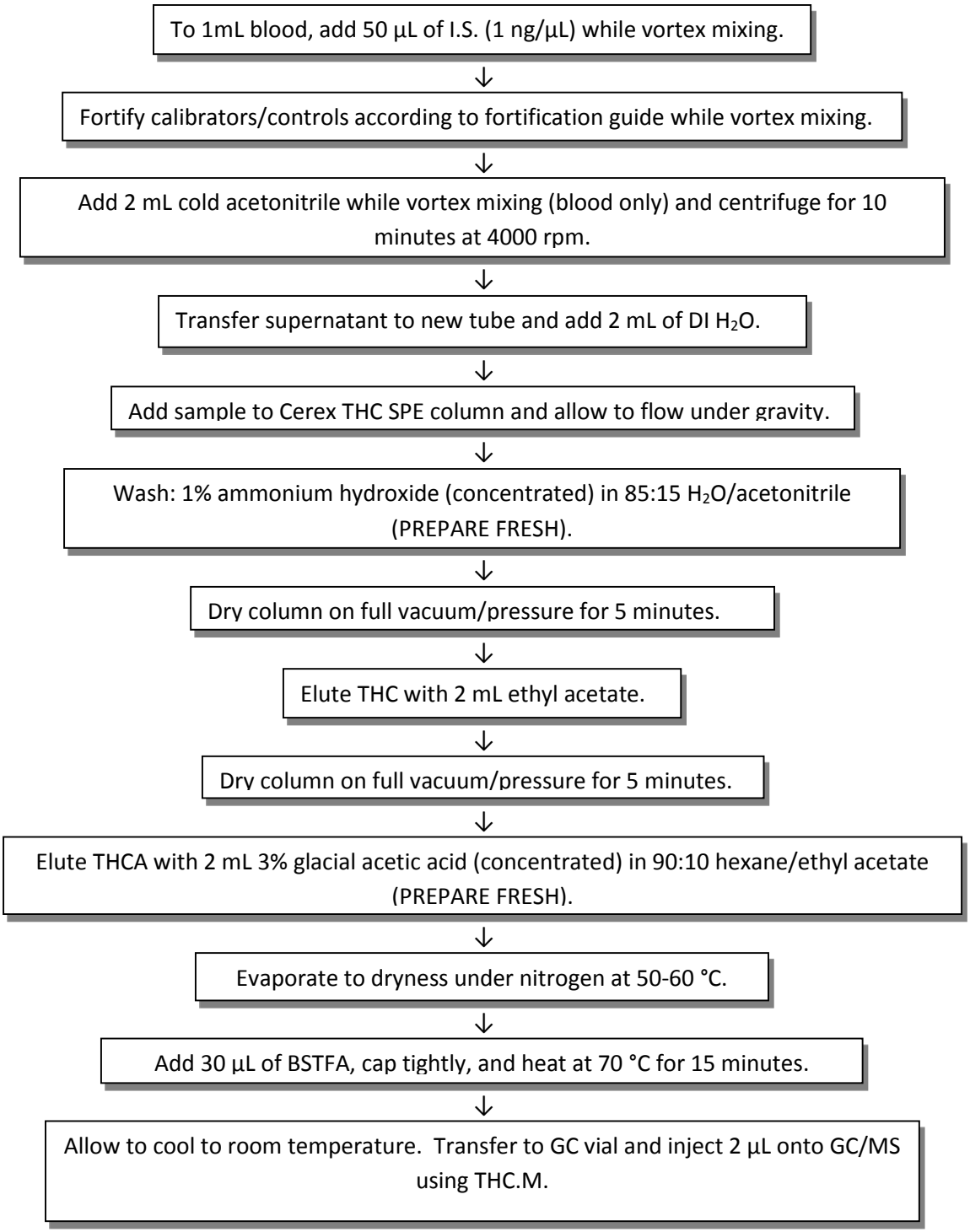
| Drug | LOD and LOQ (Blood) ng/mL | LOD (Urine) ng/mL |
|---|---|---|
| THC | 4 | 4 |
| THCA | 4 | 4 |

## 9 QUALITY CONTROLS

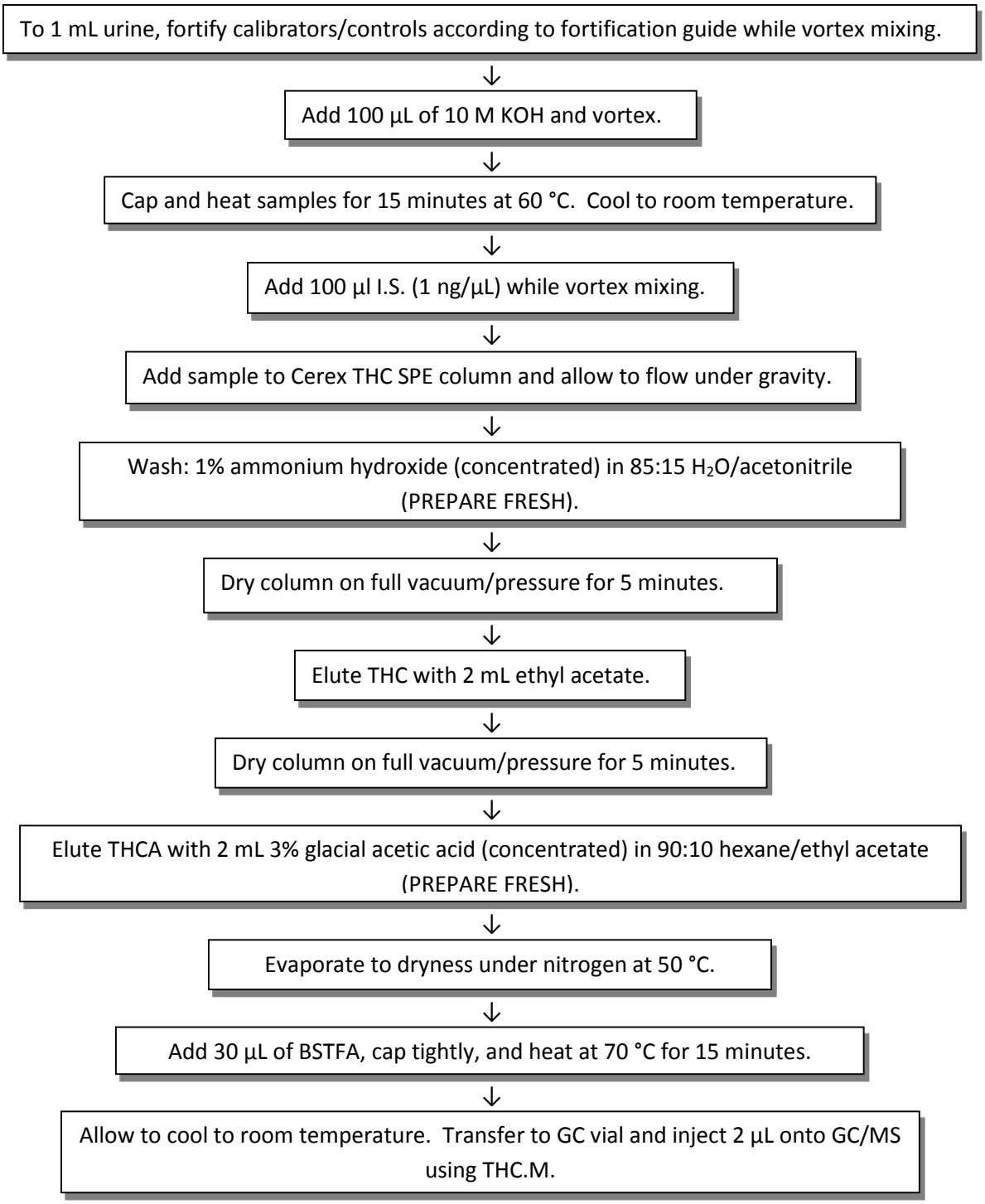
9.1 Appropriate positive and negative QCs must be included in each run. For qualitative urine analysis, each batch must contain appropriate negative and positive controls.

9.2 For quantitative analysis, use appropriate in-house or external QCs (e.g. UTAK Whole Blood QCs) when available. UTAK Drugs of Abuse Level I QC contains 50 ng/mL of THCA. Record the calculated concentrations in the appropriate QC log.

## 10 FLOWCHART FOR CANNABINOID ANALYSIS IN BLOOD


To 1mL blood, add 50 µL of I.S. (1 ng/µL) while vortex mixing.
Fortify calibrators/controls according to fortification guide while vortex mixing.
Add 2 mL cold acetonitrile while vortex mixing (blood only) and centrifuge for 10 minutes at 4000 rpm.
Transfer supernatant to new tube and add 2 mL of DI $H_2O$.
Add sample to Cerex THC SPE column and allow to flow under gravity.
Wash: 1% ammonium hydroxide (concentrated) in 85:15 $H_2O$/acetonitrile (PREPARE FRESH).
Dry column on full vacuum/pressure for 5 minutes.
Elute THC with 2 mL ethyl acetate.
Dry column on full vacuum/pressure for 5 minutes.
Elute THCA with 2 mL 3% glacial acetic acid (concentrated) in 90:10 hexane/ethyl acetate (PREPARE FRESH).
Evaporate to dryness under nitrogen at 50-60 °C.
Add 30 µL of BSTFA, cap tightly, and heat at 70 °C for 15 minutes.
Allow to cool to room temperature. Transfer to GC vial and inject 2 µL onto GC/MS using THC.M.

## 11 FLOWCHART FOR CANNABINOID ANALYSIS IN URINE


To 1 mL urine, fortify calibrators/controls according to fortification guide while vortex mixing.
Add 100 µL of 10 M KOH and vortex.
Cap and heat samples for 15 minutes at 60 °C. Cool to room temperature.
Add 100 µl I.S. (1 ng/µL) while vortex mixing.
Add sample to Cerex THC SPE column and allow to flow under gravity.
Wash: 1% ammonium hydroxide (concentrated) in 85:15 $H_2O$/acetonitrile (PREPARE FRESH).
Dry column on full vacuum/pressure for 5 minutes.
Elute THC with 2 mL ethyl acetate.
Dry column on full vacuum/pressure for 5 minutes.
Elute THCA with 2 mL 3% glacial acetic acid (concentrated) in 90:10 hexane/ethyl acetate (PREPARE FRESH).
Evaporate to dryness under nitrogen at 50 °C.
Add 30 µL of BSTFA, cap tightly, and heat at 70 °C for 15 minutes.
Allow to cool to room temperature. Transfer to GC vial and inject 2 µL onto GC/MS using THC.M.

## 12 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | THC/THCA Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 5 | 0.1 | 50 |
| 1 | 10 | 0.1 | 100 |
| 1 | 25 | 1.0 | 25 |
| 1 | 50 | 1.0 | 50 |
| 1 | 100 | 1.0 | 100 |
| 1 | 250 | 10.0 | 25 |
| 1 | 500 | 10.0 | 50 |

## 13 LITERATURE AND SUPPORTING DOCUMENTATION

13.1 Huestis, MA. Cannabis in Principles of Forensic Toxicology, 3rd Edition. Levine B (Ed). AACC Press, Washington DC, 2009, pp 269-303.

13.2 Baselt, RC. Tetrahydrocannabinol in *Disposition of Toxic Drugs and Chemicals in Man*, *8th Edition* Foster City, CA. Biomedical Publications. 2009, pp 1513-1518

13.3 Watanabe, Kanako: "11.2.2-Cannabinoids and their metabolites" in *Drugs and poisons in Humans. A Handbook of Practical Analysis.* Springer-Verlag Berlin Heidelberg. 2005, pp 187-194.

13.4 Abraham, TT; Lowe, TH; Pirnay, SO; Darwin, WD; Huestis, MA. Simultaneous GC-EI-MS determination of D9-Tetrahydrocannabinol, 11-Hydroxy-D9-tetrahydrocannabinool, and 11-nor-9-tetrahydrocannabinol in human urine following tandem enzyme-alkaline hydrolysis. Journal of Analytical Toxicology, 31, October 2007, pp 477-485

13.5 Method File: THC.M

# Carisoprodol/Meprobamate Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for confirmatory analysis of carisoprodol and/or meprobamate by solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS). Drugs are isolated from the matrix using an acidic/neutral extraction. Deuterated internal standards and selective ion monitoring (SIM) are used in electron ionization (EI) mode.

## 2 SCOPE

This procedure should be used for confirmatory analysis of carisoprodol and/or meprobamate from toxicology specimens, typically blood. This procedure is recommended if quantitative analysis is desired. Qualitative results may also be reported. For routine full scan qualitative analysis of carisoprodol and/or meprobamate, however, refer to Basic, Acidic and Neutral Drugs Confirmation by GC/MS).

## 3 REAGENTS AND STANDARDS

3.1 Carisoprodol/Meprobamate Working Standards: (Note: The concentration of carisoprodol in the working standard is half that of the meprobamate.)
Carisoprodol (0.02 mg/mL)/Meprobamate (0.04 mg/mL) Working Standard: Add 200 µL of carisoprodol (1.0 mg/mL) and 400 µL of meprobamate (1.0 mg/mL) to a 10 mL volumetric flask, and bring to volume with methanol. Store refrigerated (6 month expiration).

3.2 Internal Standard Solution: This solution consists of carisoprodol-$D_7$ and meprobamate-$D_7$ in methanol. The final concentration is 10 ng/µL.
Working Internal Standard Solution (10 ng/µL): Add 1000 µL of carisoprodol-$D_7$ (0.1 mg/mL) and 1000 µL meprobamate-$D_7$ (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.3 100 mM sodium Phosphate Buffer pH 6.0

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)
4.2 Cerex PolyChrom Clin II SPE columns
4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold
4.4 Pan balance
4.5 pH meter
4.6 Evaporator
4.7 Vortex mixer
4.8 Centrifuge
4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.3 mL/min with an injection volume of 2 µL in split mode (10:1).

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 140 °C<br>Hold for 0.5 minutes<br>30 °C/min to 290 °C<br>Hold for 2.5 minutes<br>50 °C/min to 310 °C<br>Hold for 2.6 minutes |
| Total Run Time: | 11 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

Blood and All Other Matrices

6.1 In a round bottom glass culture tube, add 0.25 mL specimen and 2 mL of 100 mM sodium phosphate buffer (pH 6.0). Vortex mix.

6.2 Add 100 µL of Working Internal Standard Solution (10 ng/µL) while vortex mixing. The final concentration of internal standard in each sample is 4 µg/mL or 4 mg/L.

6.3 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. The typical calibration range for quantitative analysis is 0-20 mg/L carisoprodol and 0-40 mg/L meprobamate. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME, ADD BUFFER AS THE DILUENT AND DOCUMENT IN THE CASE FOLDER.

6.4 A quantitative QC containing 4 mg/L carisoprodol and 8 mg/L meprobamate must be used. Use appropriate external controls (e.g., UTAK Whole Blood Controls) if available.

6.5 Centrifuge samples for at least 10 minutes at 4000 rpm.

6.6 Place collection tubes or reservoir (for waste) into vacuum manifold rack.

6.7 Add the sample to Polychrom Clin II Column. Draw the sample through using sufficient vacuum as necessary.

6.8 Wash column with 1 mL of deionized water.

6.9 Wash column with 1 mL of 1M acetic acid.

6.10 Dry column on full vacuum/pressure for 5 minutes. .

6.11 Wash column with 1 mL of hexane.
6.12 Elute drugs with 1 mL of ethyl acetate.
6.13 Turn on vacuum for a few seconds to ensure all elution solvent has drained from the column.
6.14 Remove tubes from rack and evaporate to dryness (approximately 10 minutes) under nitrogen at 50-60°C.
6.15 Reconstitute in 40 µL of ethyl acetate, vortex, and transfer to autosampler vials. An appropriate volume of solvent should be used to prevent overloading.
6.16 Inject 2 µL onto the GC/MS using CARISO-MEPRO.M.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.
7.2 SIM acquisition: CARIO-MEPRO.M. Use this method to perform targeted analysis for carisoprodol and meprobamate.

| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Meprobamate | 144 | 83, 114 | 4.16 |
| Carisoprodol | 158 | 245, 184 | 4.43 |
| Meprobamate-$D_7$ | 151 | 89, 121 | 4.13 |
| Carisoprodol-$D_7$ | 165 | 252, 191 | 4.41 |
| *Retention Time (RT) varies with column length. Deuterated (7) internal standards are used throughout. Corresponding ions for internal standards are M+7. Refer to attached method for acquisition parameters. | | | |

## 8 INTERPRETATION OF RESULTS

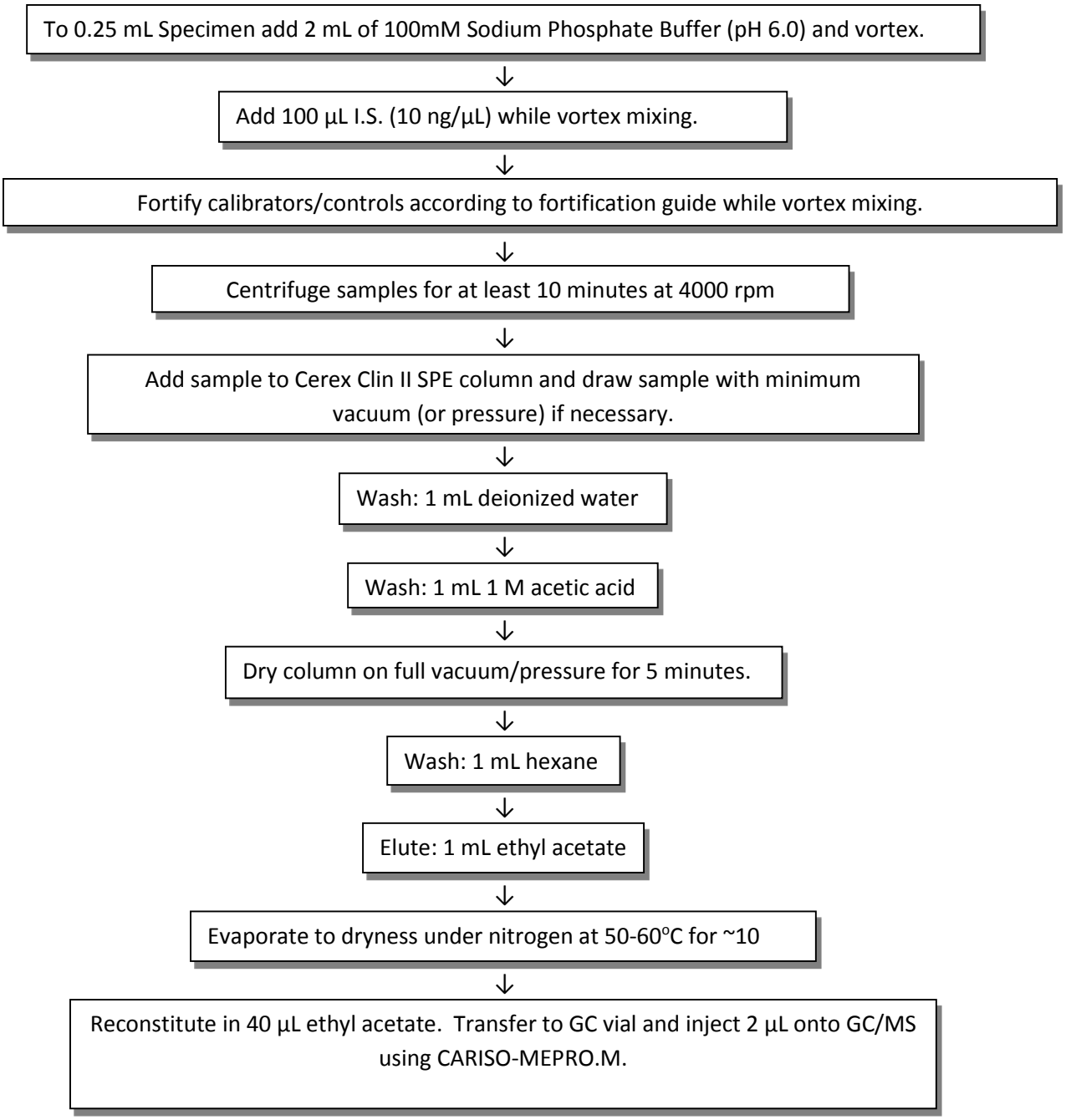
8.1 Assay performance including but not limited to prevision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.
8.2 Limits of detection in blood and urine are as follows:

| Drug | LOD and LOQ (Blood) mg/L |
|---|---|
| Meprobamate | 1.0 |
| Carisoprodol | 0.5 |

8.3 In the event of a co-extractive interference that influences the ion ratios of the internal standard (meprobamate-D7) it is permissible to use the second internal standard (carisoprodol-D7) to

report meprobamate subject to review and approval by the Laboratory Manager. In doing so, all qualitative and quantitative reporting criteria must be met.

## 9 FLOWCHART FOR CARISOPRODOL/MEPROBAMATE DETERMINATION


To 0.25 mL Specimen add 2 mL of 100mM Sodium Phosphate Buffer (pH 6.0) and vortex.
Add 100 µL I.S. (10 ng/µL) while vortex mixing.
Fortify calibrators/controls according to fortification guide while vortex mixing.
Centrifuge samples for at least 10 minutes at 4000 rpm
Add sample to Cerex Clin II SPE column and draw sample with minimum vacuum (or pressure) if necessary.
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes.
Wash: 1 mL hexane
Elute: 1 mL ethyl acetate
Evaporate to dryness under nitrogen at 50-60°C for ~10
Reconstitute in 40 µL ethyl acetate. Transfer to GC vial and inject 2 µL onto GC/MS using CARISO-MEPRO.M.

## 10 FORTIFICATION GUIDE

| Volume of Blood (mL) | Carisoprodol Target Conc. (mg/L) | Meprobamate Target Conc. (mg/L) | Carios./Mepro. Work. Std. Conc. (mg/mL) | Volume Added (µL) |
|---|---|---|---|---|
| 0.25 | 2 | 4 | 0.02/0.04 | 25 |
| 0.25 | 5 | 10 | 0.02/0.04 | 62.5 |
| 0.25 | 10 | 20 | 0.02/0.04 | 125 |
| 0.25 | 20 | 40 | 0.02/0.04 | 250 |

## 11 LITERATURE AND SUPPORTING DOCUMENTATION

11.1 Downey, Delisa and Kerrigan, Sarah. Quantitative Analysis of Carisoprodol and Meprobamate in Whole Blood Using Benzylcarbamate and Deuterated Meprobamate as Internal Standards. J Anal Toxicol 2009 June; Vol. 33, pp. 278-282.

11.2 Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 7th Ed., Biomedical Publications, Foster City, CA, p 182-183 (2004).

11.3 Method file: CARISO-MEPRO.M.

# Fentanyl Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for confirmatory analysis of fentanyl by solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS). Fentanyl is isolated from the matrix using a basic extraction. Deuterated internal standard and selective ion monitoring (SIM) are used in electron impact (EI) mode.

## 2 SCOPE

Confirmatory analysis of fentanyl from toxicology specimens including but not limited to blood. This procedure is recommended if quantitative analysis is desired. Qualitative results may also be reported. For routine full scan qualitative analysis, however, refer to Basic, Acidic, and Neutral Drugs by GC/MS.

## 3 REAGENTS AND STANDARDS

3.1 Fentanyl Working Standards

3.1.1 Preparation of 1 ng/µL Fentanyl Working Standard: Add 100 µL of Fentanyl (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.1.2 Preparation of 0.1 ng/µL Fentanyl Working Standard: Add 1 mL of Fentanyl (1 ng/µL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.1.3 Preparation of (0.5 ng/µL) Internal Working Solution: Add 50 µL of fentanyl-$D_5$, deuterated drug standard (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2 1M Acetic Acid

3.3 100 mM, pH 6.0 Phosphate Buffer

3.4 Elution Solvent: 2% ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol

3.5 Methylene Chloride/Isopropanol (80:20)

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)

4.2 Cerex PolyChrom Clin II SPE columns

4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold

4.4 Pan balance

4.5 pH meter

4.6 Evaporator

4.7 Vortex mixer

4.8 Centrifuge

4.9 Tissue homogenizer

4.10 Sonicator

4.11 Heating block

4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.3 mL/min with an injection volume of 2 µL in split mode (10:1).

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 140 °C<br>Hold for 0.5 minutes<br>30 °C/min to 290 °C<br>Hold for 2.5 minutes<br>50 °C/min to 310 °C<br>Hold for 2.6 minutes |
| Total Run Time: | 11 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

Blood, Urine, and all Other matrices

6.1 In a round bottom glass culture tube, add 2 mL of sample. NOTE IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME, ADD BUFFER AS THE DILUENT AND DOCUMENT IN THE CASE FOLDER.

6.2 Add 40 µL of Internal Standard (0.5 ng/µL) while vortex mixing. Final concentration of internal standard in each sample is 10 ng/mL.

6.3 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. Use appropriate external controls (e.g., UTAK Whole Blood Controls) when available. It is recommended the controls be at 10 ng/mL.

6.4 Add 4 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex.

6.5 Centrifuge the samples for at least 10 minutes at 4000 rpm.

6.6 Place collection tubes or reservoir (for waste) into vacuum manifold rack or positive pressure apparatus.

6.7 Pour sample onto Polychrom Clin II columns. Use sufficient vacuum/pressure to draw samples through the columns.

6.8 Wash column with 1 mL deionized water.

6.9 Wash column with 1 mL 1 M acetic acid.

6.10 Dry column on full vacuum/pressure for 5 minutes. .
6.11 Wash column with 1 mL hexane.
6.12 Wash column with 1 mL ethyl acetate.
6.13 Wash column with 1 mL methanol.
6.14 Dry column on full vacuum/pressure for 5 minutes. .
6.15 Replace waste tubes/reservoir with conical collection tubes. If the Teflon inserts (vacuum manifold only) appear dirty, replace them.
6.16 Elute basic drugs with 1 mL elution solvent (2% ammonium hydroxide in methylene chloride/isopropyl alcohol 80:20). ELUTION SOLVENT MUST BE MADE FRESH.
6.17 Turn on vacuum or apply positive pressure for a few seconds to ensure all elution solvent has drained from the column.
6.18 Evaporate to dryness under nitrogen for approximately 10 minutes at 50- 60 °C.
6.19 Reconstitute in 30 µL ethyl acetate (or appropriate volume), vortex, and transfer to autosampler vials.
6.20 Inject 2 µL onto the GC/MS using FENTANYL.M.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.

7.2 SIM acquisition: FENTANYL.M.

| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Fentanyl | 245 | 146, 189 | 7.73 |
| Fentanyl – d5 | 250 | 151, 194 | 7.71 |

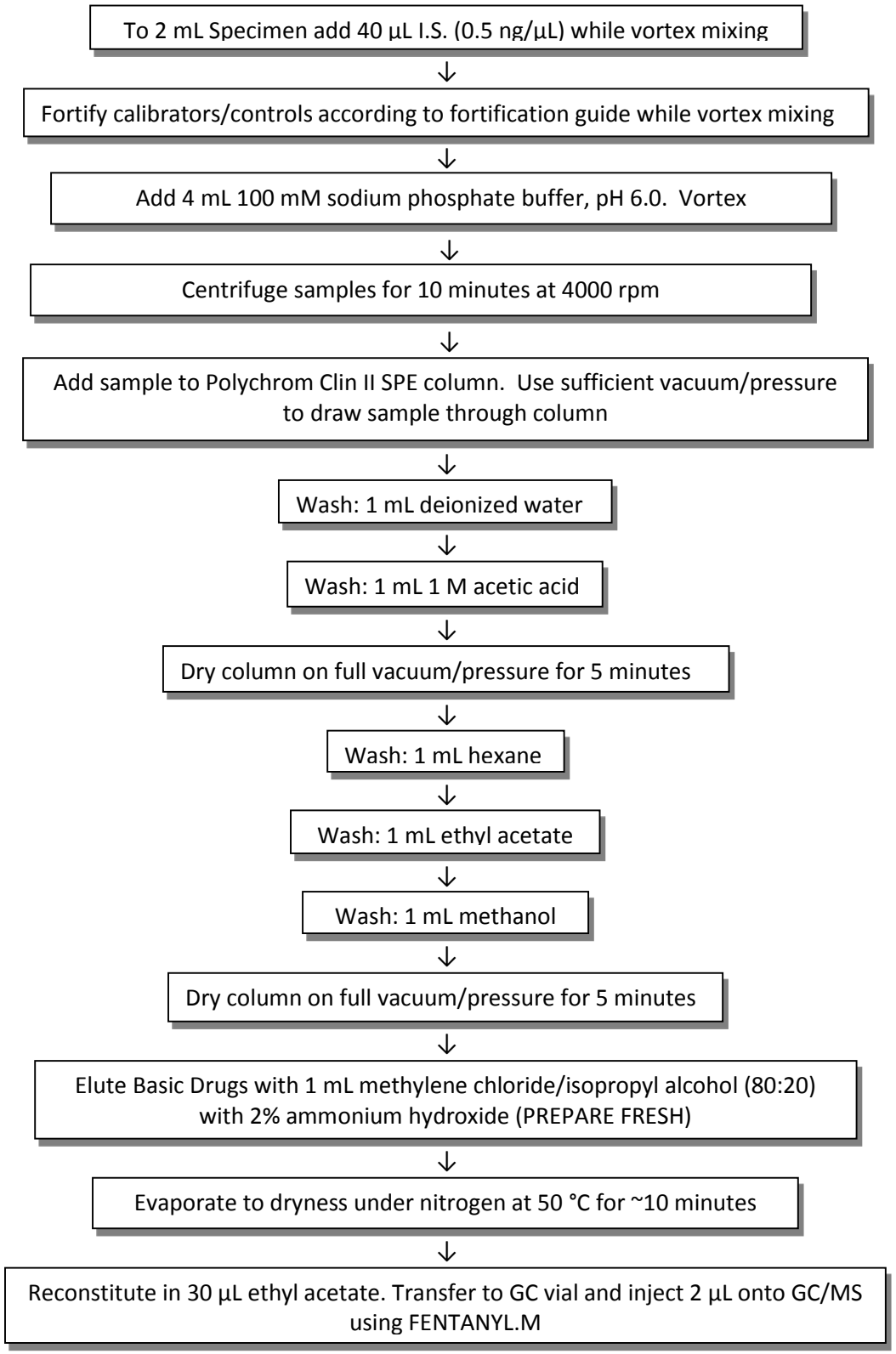
*Retention Time (RT) varies with column length. Deuterated (D5) internal standards are used throughout. Corresponding ions for internal standards are M+5. Refer to attached method for acquisition parameters.

## 8 INTERPRETATION OF RESULTS

8.1 Assay performance including but not limited to precision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.

8.2 The limit of detection and quantitation in whole blood and urine is 2.5 ng/mL.

## 9 FLOWCHART FOR FENTANYL ANALYSIS


To 2 mL Specimen add 40 µL I.S. (0.5 ng/µL) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 4 mL 100 mM sodium phosphate buffer, pH 6.0. Vortex
Centrifuge samples for 10 minutes at 4000 rpm
Add sample to Polychrom Clin II SPE column. Use sufficient vacuum/pressure to draw sample through column
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes
Wash: 1 mL hexane
Wash: 1 mL ethyl acetate
Wash: 1 mL methanol
Dry column on full vacuum/pressure for 5 minutes
Elute Basic Drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH)
Evaporate to dryness under nitrogen at 50 °C for ~10 minutes
Reconstitute in 30 µL ethyl acetate. Transfer to GC vial and inject 2 µL onto GC/MS using FENTANYL.M

## 10 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 2 | 2.5 | 0.1 | 50 |
| 2 | 5 | 0.1 | 100 |
| 2 | 10 | 0.1 | 200 |
| 2 | 25 | 1 | 50 |
| 2 | 50 | 1 | 100 |
| 2 | 100 | 1 | 200 |

## 11 LITERATURE AND SUPPORTING DOCUMENTATION

11.1 Baselt, R.C. Fentanyl. Disposition of Toxic Drugs and Chemicals in Man. 8th edition. Biomedical Publications, Foster City, CA, pp 616-619.

11.2 Bronwyn, F. et. al. Determination of subnanogram concentrations of Fentanyl in plasma by gas chromatography-mass spectrometry: comparison with standard radioimmunoassay. J Chrom B. 1997. 688, pp. 79-85.

11.3 Carson, H.J. et. al. A fatality involving an unusual route of Fentanyl delivery: Chewing and aspirating the transdermal patch. Legal Med. 2010. Vol. 12, pp. 157-159.

11.4 Kuhlman, J.J. Jr., et al. Fentanyl use, misuse, and abuse: a summary of 23 postmortem cases. J Anal Toxicol 2003; Vol. 27, pp. 499-504.

11.5 Watts, V. et al. Determination of Fentanyl in Whole Blood at Subnanogram Concentrations by Dual Capillary Column Gas Chromatography with Nitrogen Sensitive Detectors and Gas Chromatography/Mass Spectrometry. J Anal Toxicol 1988 October; Vol. 12, pp. 246-254.

11.6 Method file: FENTANYL.M

# Ketamine Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for confirmatory analysis of ketamine by solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS). Drugs are isolated from the matrix using a basic extraction. A deuterated internal standard and selective ion monitoring (SIM) are used in electron ionization (EI) mode.

## 2 SCOPE

This procedure should be used for confirmatory analysis of ketamine from toxicology specimens, typically blood. This procedure is recommended if quantitative analysis is desired. Qualitative results may also be reported. For routine full scan qualitative analysis, however, refer to Basic, Acidic and Neutral Drugs Confirmation by GC/MS.

## 3 REAGENTS AND STANDARDS

3.1 Ketamine Working Standard

3.1.1 10 ng/µL Ketamine Working Standard: Add 100 µL of ketamine (1.0 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.1.2 1 ng/µL Ketamine Working Standard: Add 1 mL of 10 ng/µL working standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2 Ketamine-D4 Internal Standard Solution

3.2.1 10 ng/µL Ketamine-D4 Stock Internal Standard: Add 1 mL of Ketamine-$D_4$ (100 µg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (12 month expiration).

3.2.2 Preparation of 1 ng/µL Ketamine Working Internal Standard: Add 1 mL of 10 ng/µL internal standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.3 1M Acetic Acid

3.4 100 mM pH 6.0 Phosphate Buffer

3.5 Methylene Chloride/Isopropanol (80:20)

3.6 Elution Solvent: 2% ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)

4.2 Cerex PolyChrom Clin II SPE columns

4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold

4.4 Pan balance

4.5 pH meter

4.6 Evaporator

4.7 Vortex mixer

4.8 Centrifuge
4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.2 mL/min with an injection volume of 2 µL in split mode (10:1).

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 140 °C<br>Hold for 0.5 minutes<br>30 °C/min to 290 °C<br>Hold for 2.5 minutes<br>50 °C/min to 310 °C<br>Hold for 2.6 minutes |
| Total Run Time: | 11 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for auto sampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

**BLOOD, URINE AND OTHER MATRICES**

6.1.1 In a round bottom glass culture tube, add 1 mL of specimen and 100 µL WORKING Internal Standard Solution (1 ng/µL) while vortex mixing (total concentration = 100 ng/mL). NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME, ADD BUFFER AS THE DILUENT AND DOCUMENT IN THE CASE FOLDER.

6.1.2 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. A minimum of one in-house control should be included in each run. Use appropriate external controls (e.g., UTAK Whole Blood Controls) when available. An in-house quantitative QC of 100 ng/mL is recommended.

6.1.3 Add 2 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex.

6.1.4 Centrifuge samples for at least 10 minutes at 4000 rpm.

6.1.5 Place collection tubes or reservoir (for waste) into vacuum manifold rack or positive pressure apparatus.

6.1.6 Pour sample onto Polychrom Clin II columns. Use sufficient vacuum/pressure to draw samples through the columns.
6.1.7 Wash column with 1 mL deionized water.
6.1.8 Remove waste tubes/reservoir and replace with fresh waste tubes/reservoir.
6.1.9 Wash column with 1 mL 1 M acetic acid.
6.1.10 Dry column on full vacuum/pressure for 5 minutes.
6.1.11 Wash column with 1 mL hexane.
6.1.12 Wash column with 1 mL ethyl acetate.
6.1.13 Wash column with 1 mL methanol.
6.1.14 Dry column on full vacuum/pressure for 5 minutes.
6.1.15 Replace waste tubes/reservoir with conical collection tubes. If the Teflon inserts (vacuum manifold only) appear dirty, replace them.
6.1.16 Elute basic drugs with 1mL elution solvent (2% ammonium hydroxide in methylene chloride/isopropyl alcohol 80:20). ELUTION SOLVENT MUST BE MADE FRESH.
6.1.17 Turn on vacuum or apply positive pressure for a few seconds to ensure all elution solvent has drained from the column.
6.1.18 Evaporate to dryness under nitrogen for approximately 10 minutes at 50-60 °C. Reconstitute in 30µL ethyl acetate (or an appropriate volume), vortex, and transfer to autosampler vials.
6.1.19 Inject 2 µL onto the GC/MS using KETAMINE.M. **(FOR URINE SAMPLES ONLY USE—KETAMINE_URINE.M)**

6.2 Dilutions: If the sample is known to contain a high concentration of drug, it should be diluted prior to analysis. Aliquot the desired volume of blood and make up to volume using phosphate buffer. DOCUMENT THESE CHANGES IN THE CASE FOLDER. Then proceed from step 4 of the blood procedure.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.
7.2 SIM acquisition: **KETAMINE_URINE.M.** Use this method to perform targeted analyses for ketamine in urine.
7.3 SIM acquisition: **KETAMINE.M.** Use this method to perform targeted analyses for ketamine in blood.

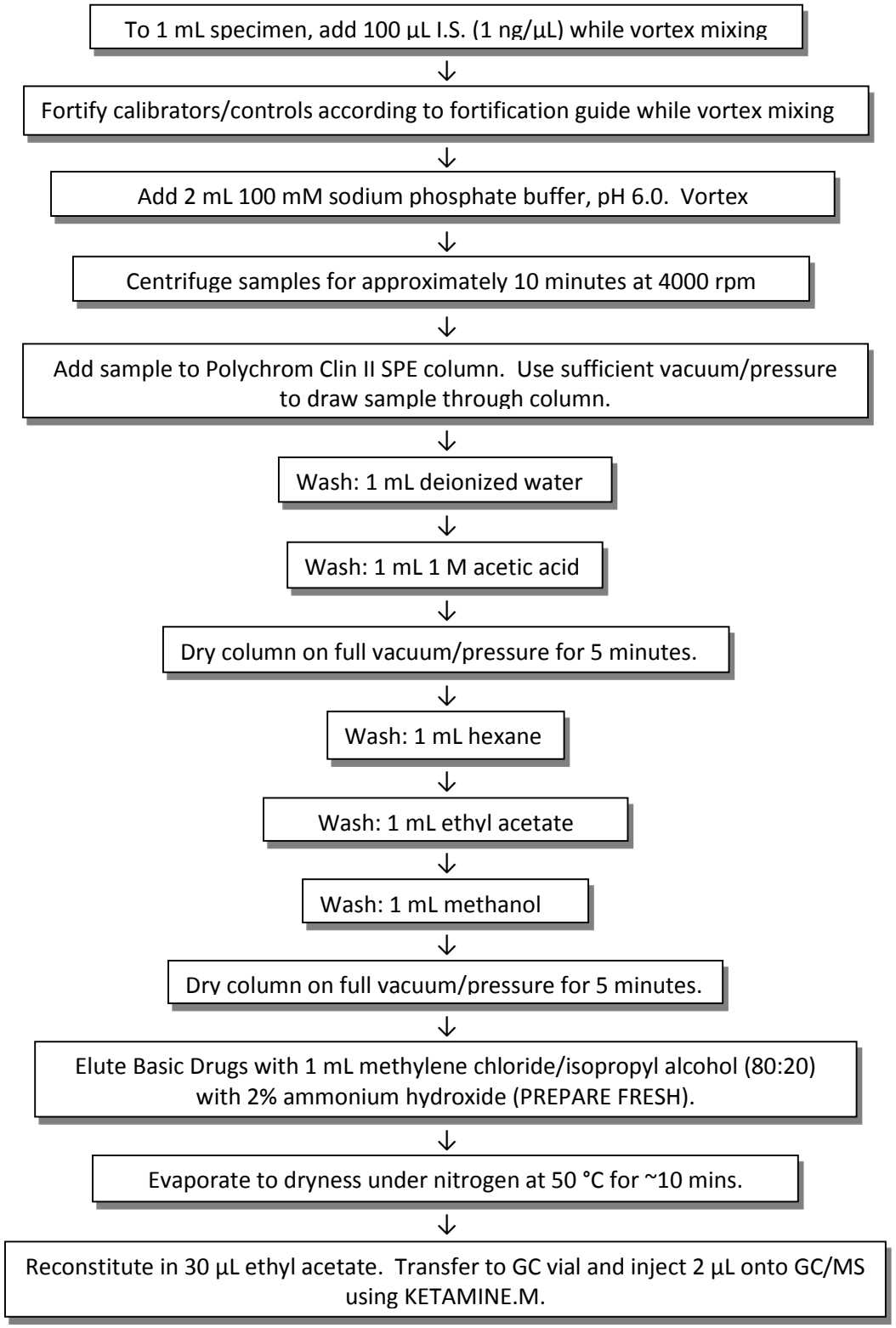
| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Ketamine-D4 | 213 | 184, 156 | 4.46 |
| Ketamine | 209 | 180, 152 | 4.47 |
| *Retention Time (RT) varies with column length. Deuterated (D4) internal standards are used throughout. Corresponding ions for internal standards are M+4. Refer to attached method for acquisition parameters. | | | |

## 8 INTERPRETATION OF RESULTS

8.1 Assay performance including but not limited to precision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.

8.2 Limits of detection and quantitation of ketamine in blood are 10 ng/mL.

## 9 FLOWCHART FOR KETAMINE ANALYSIS


To 1 mL specimen, add 100 µL I.S. (1 ng/µL) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 2 mL 100 mM sodium phosphate buffer, pH 6.0. Vortex
Centrifuge samples for approximately 10 minutes at 4000 rpm
Add sample to Polychrom Clin II SPE column. Use sufficient vacuum/pressure to draw sample through column.
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes.
Wash: 1 mL hexane
Wash: 1 mL ethyl acetate
Wash: 1 mL methanol
Dry column on full vacuum/pressure for 5 minutes.
Elute Basic Drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH).
Evaporate to dryness under nitrogen at 50 °C for ~10 mins.
Reconstitute in 30 µL ethyl acetate. Transfer to GC vial and inject 2 µL onto GC/MS using KETAMINE.M.

## 10 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 20 | 1 | 20 |
| 1 | 50 | 1 | 50 |
| 1 | 100 | 1 | 100 |
| 1 | 250 | 10 | 25 |
| 1 | 500 | 10 | 50 |
| 1 | 1000 | 10 | 100 |

## 11 LITERATURE AND SUPPORTING DOCUMENTATION

11.1 Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 7th Ed., Biomedical Publications, Foster City, CA, p 586-588 (2004).

11.2 Jenkins, Amanda J. Hallucinogens *in* Principles of Forensic Toxicology, 2nd Ed., Levine, Barry, Ed. AACC Press, Washington DC, pp 265-283 (2003).

11.3 Method file: KETAMINE.M

11.4 Method file: KETAMINE_URINE.M

# Keto Opioids Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for confirmatory analysis of hydrocodone, hydromorphone, oxycodone, and oxymorphone using solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS). Drugs are isolated from the matrix using a basic extraction. Deuterated internal standards and selective ion monitoring (SIM) are used in electron impact (EI) mode.

## 2 SCOPE

Confirmatory analysis of keto opioids from toxicology specimens, including but not limited to blood. Urine confirmations are reported only qualitatively and may also be reported using Basic, Acidic and Neutral Drugs Confirmation by GC/MS.

## 3 REAGENTS AND STANDARDS

3.1 Keto Opioid Working Standard

3.1.1 Two solutions will be needed for this analysis. The solutions contain concentrations of hydrocodone, hydromorphone, oxycodone, and oxymorphone at 10 ng/µL and 1 ng/µL, respectively.

3.1.2 Preparation of 10 ng/µL Keto Opioid Working Standard: Add 100 µL of each keto opioid (1.0 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.1.3 Preparation of 1 ng/µL Working Standard: Add 1 mL of 10 ng/µL working standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2 Keto Opioid Internal Standard Solution: This solution contains concentrations of hydrocodone-$D_3$, hydromorphone-$D_3$, oxycodone-$D_3$, and oxymorphone-$D_3$.

3.2.1 Preparation of 10 ng/µL Keto Opioid Stock Internal Standard: Add 100 µL of each keto opioid (100 µg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2.2 Preparation of 1 ng/µL Working Internal Standard: Add 1 mL of 10 ng/µL internal standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.3 1M Acetic Acid

3.4 100 mM, pH 6.0 Phosphate Buffer

3.5 Methylene Chloride/Isopropanol (80:20)

3.6 Elution Solvent: 2% ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol

3.7 Acetic anhydride (GC Grade) for derivatization. Store at room temperature.

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)

4.2 Cerex PolyChrom Clin II SPE columns
4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold
4.4 Pan balance
4.5 pH meter
4.6 Evaporator
4.7 Vortex mixer
4.8 Centrifuge
4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.2 mL/min with an injection volume of 2 µL in split mode (10:1).

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 140 °C<br>Hold for 0.5 minutes<br>30 °C/min to 290 °C<br>Hold for 2.5 minutes<br>50 °C/min to 310 °C<br>Hold for 1.6 minutes |
| Total Run Time: | 10 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

Blood, Urine, and all Other Matrices

6.1 In a round bottom glass culture tube, add 1 mL of specimen and 100 µL WORKING Internal Standard Solution (1 ng/µL) while vortex mixing (total concentration = 100 ng/mL). NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME AND DOCUMENT IN THE CASE FOLDER.

6.2 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. Use appropriate external controls (e.g. UTAK Whole Blood Controls) when available.

6.3 Add 2 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex.

6.4 Place collection tubes or reservoir (for waste) into vacuum manifold rack or positive pressure apparatus.

6.5 Pour sample onto Polychrom Clin II columns. Use sufficient vacuum/pressure to draw samples through the columns.

6.6 Wash column with 1 mL deionized water.

6.7 Remove waste tubes/reservoir and replace with fresh waste tubes/reservoir.

6.8 Wash column with 1 mL 1 M acetic acid.

6.9 Dry column on full vacuum/pressure for 5 minutes.

6.10 Wash column with 1 mL hexane.

6.11 Wash column with 1 mL ethyl acetate.

6.12 Wash column with 1 mL methanol.

6.13 Dry column on full vacuum/pressure for 5 minutes.

6.14 Replace waste tubes/reservoir with conical collection tubes. If the Teflon inserts (vacuum manifold only) appear dirty, replace them.

6.15 Elute basic drugs with 1 mL elution solvent (2% ammonium hydroxide in methylene chloride/isopropyl alcohol 80:20). ELUTION SOLVENT MUST BE MADE FRESH DAILY.

6.16 Turn on vacuum or apply positive pressure for a few seconds to ensure all elution solvent has drained from the column.

6.17 Evaporate to dryness under nitrogen for 15 minutes at 60 °C.

6.18 Reconstitute in 10µL ethyl acetate and 10 µL acetic anhydride (or other appropriate volume of 1:1 ethyl acetate/acetic anhydride), vortex, and transfer to autosampler vials.

6.19 Inject 2 µL onto the GC/MS using KETO.M.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.

7.2 SIM acquisition: KETO.M.

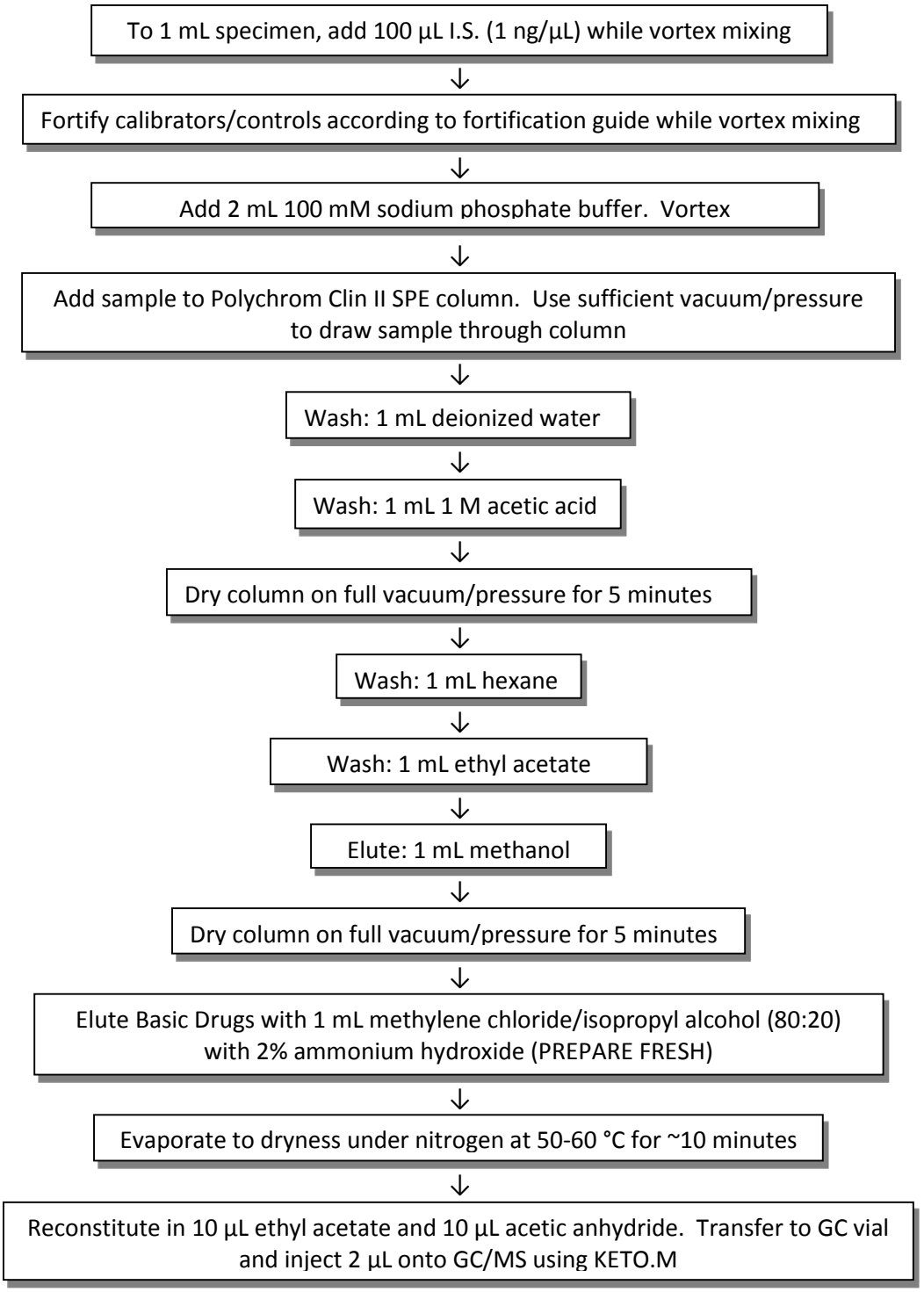
| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Hydrocodone-d3 | 302 | 273, 287 | 6.541 |
| Hydrocodone | 299 | 270, 284 | 6.550 |
| Hydromorphone-d3 | 288 | 259, 330 | 7.178 |
| Hydromorphone | 285 | 256, 327 | 7.188 |
| Oxycodone-d3 | 360 | 301, 317 | 6.876 |
| Oxycodone | 357 | 298, 314 | 6.889 |
| Oxymorphone-d3 | 346 | 303, 388 | 7.472 |
| Oxymorphone | 343 | 300, 385 | 7.482 |
| *Retention Time (RT) varies with column length. Deuterated (D3) internal standards are used throughout. Corresponding ions for internal standards are M+3. | | | |

## 8 INTERPRETATION OF RESULTS

Assay performance including but not limited to precision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.

| **Drug** | **LOD and LOQ in Blood ng/mL** | LOD in Urine ng/mL |
|---|---|---|
| Hydrocodone | 20 | 30 |
| Hydromorphone | 25 | 20 |
| Oxycodone | 20 | 20 |
| Oxymorphone | 25 | 20 |

## 9 FLOWCHART FOR KETO OPIOIDS ANALYSIS.


To 1 mL specimen, add 100 µL I.S. (1 ng/µL) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 2 mL 100 mM sodium phosphate buffer. Vortex
Add sample to Polychrom Clin II SPE column. Use sufficient vacuum/pressure to draw sample through column
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes
Wash: 1 mL hexane
Wash: 1 mL ethyl acetate
Elute: 1 mL methanol
Dry column on full vacuum/pressure for 5 minutes
Elute Basic Drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH)
Evaporate to dryness under nitrogen at 50-60 °C for ~10 minutes
Reconstitute in 10 µL ethyl acetate and 10 µL acetic anhydride. Transfer to GC vial and inject 2 µL onto GC/MS using KETO.M

## 10 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 20 | 1 | 20 |
| 1 | 50 | 1 | 50 |
| 1 | 100 | 1 | 100 |
| 1 | 250 | 10 | 25 |
| 1 | 500 | 10 | 50 |
| 1 | 1000 | 10 | 100 |

## 11 LITERATURE AND SUPPORTING DOCUMENTATION

11.1 Kerrigan S; Goldberger BA. Opioids in Principles of Forensic Toxicology, 3rd Edition. Levine B (Ed). AACC Press, Washington DC, 2009, pp 225-244.

11.2 Method file: KETO.M

# Methadone/EDDP Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for confirmatory analysis of methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) by solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS). Drugs are isolated from the matrix using a basic extraction. Deuterated internal standards and selective ion monitoring (SIM) are used in electron ionization (EI) mode.

## 2 SCOPE

Confirmatory analysis of Methadone/EDDP from toxicology specimens including but not limited to blood. This procedure is recommended if quantitative analysis is desired. Qualitative results may also be reported. For routine full scan qualitative analysis, however, refer to Basic, Acidic and Neutral Drugs Confirmation by GC/MS.

## 3 REAGENTS AND STANDARDS

3.1 Methadone/EDDP Stock Standard:

3.1.1 Preparation of 0.1 mg/mL Methadone/EDDP Stock Standard: Add 1000 µL of methadone and EDDP (1.0 mg/mL) to a 10mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.2 Methadone/EDDP Working Standards

3.2.1 Preparation of 10 ng/µL Methadone/EDDP Working Standard: Add 1000 µL of methadone (0.1 mg/mL) and 1000 µL of EDDP (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2.2 Preparation of a 1 ng/µL Methadone/EDDP Working Solution: Add 1000 µL of Methadone/EDDP (10 ng/µL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.3 Internal Standard Solution: Methadone-D9/EDDP-D3 (5 ng/µL)

3.3.1 Preparation of 5 ng/µL Internal Standard Solution: Add 500 µL of Methadone-$D_9$ and EDDP-$D_3$ (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.4 1M Acetic Acid

3.5 100 mM, pH 6.0 Phosphate Buffer

3.6 Methylene Chloride/Isopropanol (80:20)

3.7 Elution Solvent: 2% ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)

4.2 Cerex PolyChrom Clin II SPE columns

4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold

4.4 Pan balance

4.5 pH meter
4.6 Evaporator
4.7 Vortex mixer
4.8 Centrifuge
4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.3 mL/min with an injection volume of 2 µL in split mode (10:1).

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 140 °C<br>Hold for 0.5 minutes<br>30 °C/min to 290 °C<br>Hold for 2.5 minutes<br>50 °C/min to 310 °C<br>Hold for 2.6 minutes |
| Total Run Time: | 11 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

Blood, Urine, and all Other matrices

6.1 In a round bottom glass culture tube, add 1 mL of sample. NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALLER VOLUME AND DOCUMENT IN THE CASE FOLDER.

6.2 Add 50 µL of Internal Standard (5 ng/µL) while vortex mixing. Final concentration of internal standard in each sample is 250 ng/mL.

6.3 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. The typical calibration range for quantitative analysis is 0-1000 ng/mL for Methadone/EDDP. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. An in-house control containing 100 ng/mL methadone/EDDP is recommended. Use appropriate external controls (e.g. UTAK Whole Blood Controls) when available.

6.4 Add 2 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex.

6.5 Centrifuge samples for 10 minutes at 4000 rpm.

6.6 Place collection tubes or reservoir (for waste) into vacuum manifold rack or positive pressure apparatus.

6.7 Pour sample onto Polychrom Clin II columns. Use sufficient vacuum/pressure to draw samples through the columns.

6.8 Wash column with 1 mL deionized water.

6.9 Wash column with 1 mL 1 M acetic acid.

6.10 Dry column on full vacuum/pressure for 5 minutes.

6.11 Wash column with 1 mL hexane.

6.12 Wash column with 1 mL ethyl acetate.
6.13 Wash column with 1 mL methanol.
6.14 Dry column on full vacuum/pressure for 5 minutes.
6.15 Replace waste tubes/reservoir with conical collection tubes. If the Teflon inserts (vacuum manifold only) appear dirty, replace them.
6.16 Elute basic drugs with 1 mL elution solvent (2% ammonium hydroxide in methylene chloride/isopropyl alcohol 80:20). ELUTION SOLVENT MUST BE MADE FRESH.
6.17 Turn on vacuum or apply positive pressure for a few seconds to ensure all elution solvent has drained from the column.
6.18 Evaporate to dryness under nitrogen for approximately 10 minutes at 50-60°C.
6.19 Reconstitute in 30µL ethyl acetate (or appropriate volume), vortex, and transfer to autosampler vials.
6.20 Inject 2 µL onto the GC/MS using METHADONE-EDDP.M.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.

7.2 SIM acquisition: Methadone-EDDP.M.

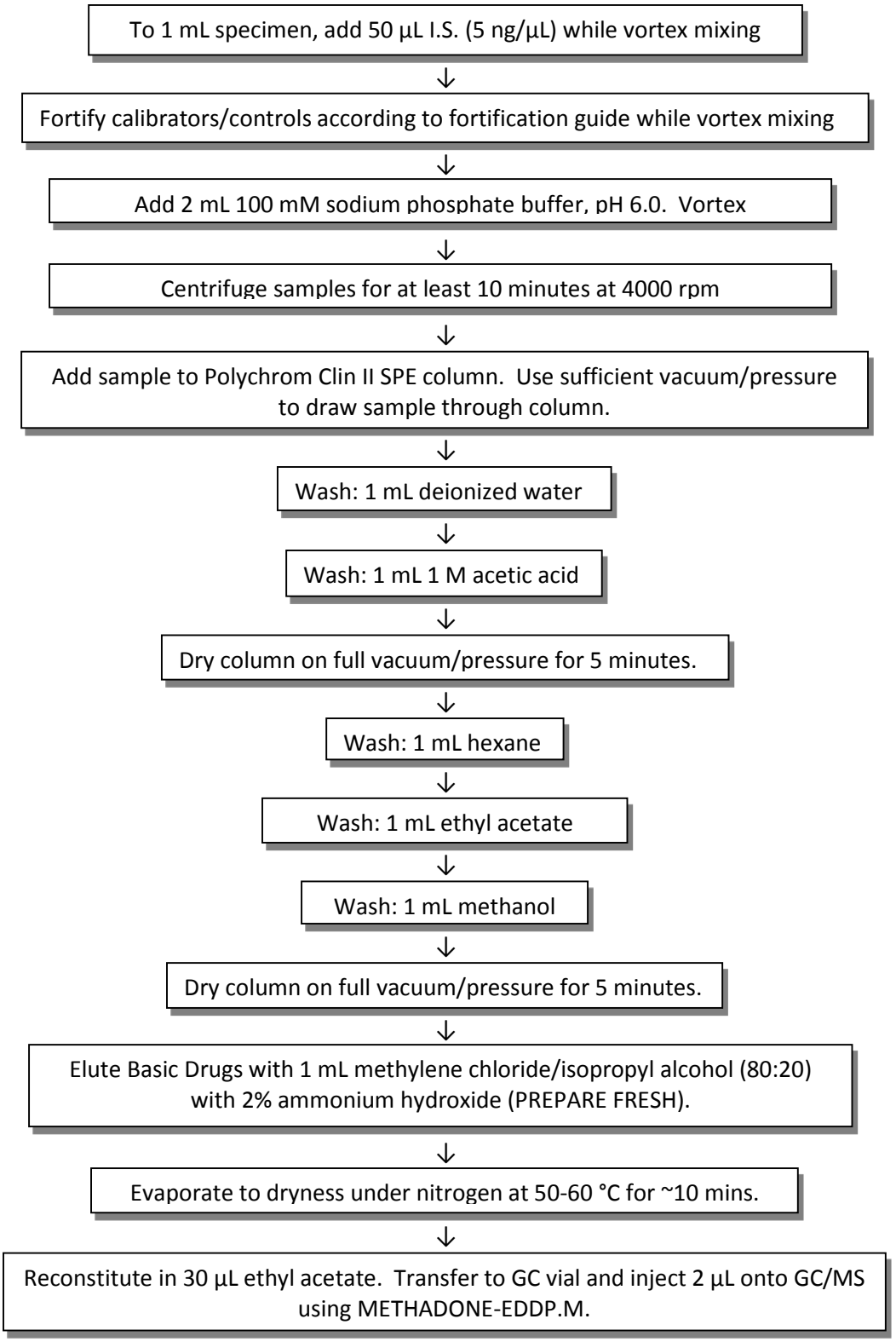
| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| EDDP | 277 | 200, 276 | 5.07 |
| EDDP-D3 | 280 | 279, 203 | 5.06 |
| Methadone | 72 | 223, 294 | 5.4 |
| | 75 | 226, 303 | 5.39 |
| *Retention Time (RT) varies with column length. Deuterated (D3 and D9) internal standards are used throughout. | | | |

## 8 INTERPRETATION OF RESULTS

8.1 Assay performance including but not limited to precision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.

8.2 The limits of detection and quantitation for methadone and EDDP are 20 ng/mL in blood.

## 9 FLOWCHART FOR METHADONE/EDDP ANALYSIS


To 1 mL specimen, add 50 µL I.S. (5 ng/µL) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 2 mL 100 mM sodium phosphate buffer, pH 6.0. Vortex
Centrifuge samples for at least 10 minutes at 4000 rpm
Add sample to Polychrom Clin II SPE column. Use sufficient vacuum/pressure to draw sample through column.
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes.
Wash: 1 mL hexane
Wash: 1 mL ethyl acetate
Wash: 1 mL methanol
Dry column on full vacuum/pressure for 5 minutes.
Elute Basic Drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH).
Evaporate to dryness under nitrogen at 50-60 °C for ~10 mins.
Reconstitute in 30 µL ethyl acetate. Transfer to GC vial and inject 2 µL onto GC/MS using METHADONE-EDDP.M.

## 10 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 20 | 1 | 20 |
| 1 | 50 | 1 | 50 |
| 1 | 100 | 1 | 100 |
| 1 | 250 | 10 | 25 |
| 1 | 500 | 10 | 50 |
| 1 | 1000 | 10 | 100 |

## 11 LITERATURE AND SUPPORTING DOCUMENTATION

11.1 Lucas, A.C.S. et al. Use of solid-phase microextraction (SPME) for the determination of methadone and EDDP in human hair by GC-MS. Forensic Science International. 2000; 107, pp. 225-232.

11.2 Nikolaou, P.D. et al. Development and Validation of an EI-GC-MS Method for the Determination of Methadone and its Major Metabolites (EDDP and EMDP) in Human Breast Milk. J Anal Toxicol 2008 Sept; Vol. 32, pp. 478-484

11.3 Nikolaou, P.D. et al. Validated method for the simultaneous determination of methadone and its main metabolites (EDDP and EMDP) in plasma of umbilical cord blood by gas chromatography-mass spectrometry. J Chrom B. 2008, 867, pp 219-225.

11.4 R.C. Baselt. Disposition of Toxic Drugs and Chemicals in Man. 8th edition. Biomedical Publications, Foster City, CA, pp 941-944.

11.5 Kuhlman, J.J. Jr., et al. Fentanyl use, misuse, and abuse: a summary of 23 postmortem cases. J Anal Toxicol 2003; Vol. 27, pp. 499-504.

11.6 Method file: Methadone-EDDP.M

# Opiates and Cocaine Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for morphine, codeine, 6-acetylmorphine (6-AM), cocaine, benzoylecgonine (BE), ecgonine methyl ester (EME), and cocaethylene (CE). Drugs are isolated from the matrix using solid phase extraction (SPE). Perfluoroacyl derivatives are prepared and analyzed using gas chromatography/mass spectrometry (GC/MS), deuterated internal standards and selective ion monitoring (SIM) in electron impact (EI) mode.

## 2 SCOPE

Confirmatory analysis of target analytes from toxicology specimens including but not limited to blood and urine. Urine confirmations are reported only qualitatively.

## 3 STANDARDS AND SOLUTIONS

3.1 Opiate and Cocaine ("OPICOC") Working Standards

Two working standards are used. Each contains morphine, codeine, 6-AM, cocaine, BE, EME and CE at either 10 ng/µL or 1 ng/µl, respectively.

3.1.1 Preparation of 10 ng/µl OPICOC Working Standard

Add 1000 µL of each drug standard (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with acetonitrile. Store refrigerated (3 month expiration).

3.1.2 Preparation of a 1 ng/µL OPICOC Working Standard

Add 1000 µL of the 10 ng/µL OPICOC working standard to a 10 mL volumetric flask and bring to volume with acetonitrile. Store refrigerated (3 month expiration).

3.2 Internal Standard Solution

This solution consists of morphine-d3, codeine-d3, cocaine-d3, BE-d3, EME-d3, CE-d3 (1ng/µL) and 6-AM (0.5 ng/µL) in acetonitrile.

3.2.1 Preparation of (10 ng/µL) Internal Standard Stock Solution

Add 1000 µL of each deuterated drug standard (0.1 mg/ml) to a 10 mL volumetric flask and bring to volume with acetonitrile. Store refrigerated (3 month expiration).

3.2.2 Preparation of (1 ng/µL) Internal Standard Solution

Add 1000 µL of 10 ng/µL internal standard stock solution to a 10 mL volumetric flask and bring to a volume with acetonitrile. Store refrigerated (3 month expiration).

3.3 0.1 M HCl

3.4 1 M HCl

3.5 Cold Acetonitrile: HPLC grade acetonitrile used in this procedure should be stored in the refrigerator at 0 °C to 10 °C prior to use.

3.6 Elution Solvent: 2% ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol

3.7 Methylene Chloride/Isopropanol (80:20)

3.8 PFPA (pentafluoropropionic anhydride) and HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) for derivatization. Store both reagents at room temperature. Discard if there is a change in color.

3.9 Deconjugation Enzyme: E-coli Type IX-A β-Glucuronidase (125,000 Units). To one vial of 125,000 units, add 25 mL of deconjugation buffer and mix gently. Store frozen (expiration of reconstituted enzyme reagent is 30 days).

3.10 Deconjugation Buffer (100 mM sodium phosphate buffer, pH 6.8)

3.11 Conjugated Morphine Working Standard (10 ng/µL): For total morphine determination only, a morphine-3-glucuronide working standard is used. Add 1000 µL of 0.1 mg/ml morphine-3-glucuronide drug standard to a 10 mL volumetric flask. Bring to volume with acetonitrile. Store refrigerated (6 month expiration).

3.12 Conjugated Morphine Internal Standard (10 ng/µL): For total morphine determination only, a deuterated morphine-3-glucuronide internal standard is used. Add 1000 µL of a 0.1 mg/ml morphine-3-glucuronide-d3 drug standard to a 10 mL volumetric flask. Bring to volume with acetonitrile. Store refrigerated (6 month expiration).

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)

4.2 Cerex PolyChrom Clin II SPE columns

4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold

4.4 Pan balance

4.5 pH meter

4.6 Evaporator

4.7 Vortex mixer

4.8 Centrifuge

4.9 Tissue homogenizer

4.10 Sonicator

4.11 Heating block

4.12 Vacuum pump or house vacuum

4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.1 mL/min with an injection volume of 2 µL in split mode (10:1)

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 100 °C<br>Hold for 0 minutes<br>30 °C/min to 230 °C<br>Hold for 5 minutes<br>30 °C/min to 290 °C<br>Hold for 5 minutes |
| Total Run Time: | 16.333 minutes |
| Injector Temperature: | 260 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

6.1 Blood – **Use silanized glassware for blood extracts**

6.1.1 In a round bottom glass centrifuge tube add 1 mL blood and 50 µL Internal Standard Solution (1 ng/µL) while vortex mixing. Final concentration of internal standard in each sample of 50 ng/mL for all target drugs except 6-MAM (25 ng/ml). NOTE: IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALL VOLUME AND DOCUMENT IN THE CASE RECORD.

6.1.2 Fortify all controls and calibrators while mixing using the appropriate amount of working standard. The typical calibration range for quantitative analysis is 0-1000 ng/mL. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. A minimum of one in-house control (100 ng/mL) should be included in each run. Use appropriate external controls (e.g., UTAK Whole Blood Controls) when available.

6.1.3 Precipitate proteins by addition of 2 mL cold acetonitrile while vortex mixing.

6.1.4 Centrifuge for 10 minutes at 4000 rpm.

6.1.5 Pour off the supernatant layer into a clean round bottom glass tube. Add 1 mL 0.1 M HCl.

6.1.6 Place collection tubes or reservoir (for waste) into vacuum manifold rack or on positive pressure apparatus.

6.1.7 Pour acidified sample onto SPE columns. Samples should flow under gravity. Do not use the vacuum (or pressure) unless it is necessary.

6.1.8 Wash column with 1 mL deionized water.

6.1.9 Wash column with 1 mL 0.1 M HCl.

6.1.10 Wash column with 1 mL methanol.

6.1.11 Wash column with 1 mL ethyl acetate.

6.1.12 Dry column on full vacuum/pressure for 5 minutes.

6.1.13 Place conical glass collection tubes into the rack.

6.1.14 Elute drugs with 1 mL elution solvent (2% ammonium hydroxide in methylene chloride/isopropyl alcohol 80:20). Samples should flow under gravity. Do not use vacuum (or pressure) unless it is necessary. ELUTION SOLVENT MUST BE MADE FRESH DAILY.

6.1.15 Turn on vacuum for a few seconds to ensure all elution solvent has drained from the column.

6.1.16 Remove tubes from rack and evaporate to dryness (approximately 10 minutes) under nitrogen at 50 °C.

6.1.17 IN THE FUME HOOD, add 75 µL PFPA and 75 µL HFIP. Cap tightly. Heat at 70 °C for 15 minutes.

6.1.18 Evaporate to dryness under nitrogen for 10 minutes at 50 °C. Reconstitute in 30 µL ethyl acetate, vortex, and transfer to autosampler vials. It is acceptable to increase the

reconstitution volume prior to injection as needed to prevent sample overload. The presumptive screening test results can be used as a guide.

6.1.19 Inject 2 µL onto the GC/MS using OPICOCSIM.M.

6.2 **URINE:**

6.2.1 In a round bottom glass centrifuge tube, add 1 mL urine and 50 µL Internal Standard Solution (1 ng/µL) while vortex mixing. Final concentration of internal standard in each sample of 50 ng/mL.

6.2.2 For qualitative analysis include a minimum of one negative and one positive control in each run. For preparation of 200 ng/mL positive control, add 20 µL of OPICOC Working Standard Solution (10 ng/µL) to 1 mL urine while vortex mixing.

6.2.3 If sample is cloudy or contains particulates, centrifuge for 10 minutes, 4000 rpm.

6.2.4 Add 1 mL 1 M HCl and mix.

6.2.5 Proceed from step 6 through 17 of the blood procedure.

6.2.6 Evaporate to dryness under nitrogen for 10 minutes at 50 °C. Reconstitute in 50 µL ethyl acetate, vortex and transfer to autosampler vials. Samples containing extremely elevated drug concentration (based on presumptive immunoassay results) may be reconstituted using a larger volume of ethyl acetate if necessary.

6.2.7 Inject 2 µL onto the GC/MS using OPICOCSIM.M.

## 7 TOTAL MORPHINE DETERMINATION

7.1 In a round bottom glass centrifuge tube, add 1 mL blood and 25 µL of 10 ng/µL morphine-3-glucuronide-d3 Internal Standard Solution to all standards, QCs, and samples. **Note**: This is a different deuterated internal standard from the free opiate analysis. Deuteration and glucuronidation of the internal standard ensures that the deconjugation of opiates in the sample is monitored.

7.2 Fortify all controls and calibrators while vortex mixing using the appropriate amount of conjugated morphine working standard. Refer to the fortification guide for guidance on preparation of calibrators. A minimum of one in-house control should be included in each run. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS.

7.3 Add 0.2 mL deconjugation enzyme (5000 units). Incubate at 37 °C for 3 hours.

7.4 Proceed from step 3 of the blood procedure.

## 8 INSTRUMENTAL ANALYSIS

8.1 Ensure that the daily QC and tune verification have been completed.

8.2 SIM acquisition: OPICOCSIM.M. Use this method to perform targeted analysis for morphine, codeine, 6-AM, cocaine, BE, EME and CE.

| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Morphine | 577 | 558, 414 | 9.43 |
| Codeine | 445 | 282, 266 | 9.97 |
| 6-AM | 414 | 473, 361 | 10.60 |
| Cocaine | 303 | 182, 82 | 8.70 |
| BE | 439 | 318, 272 | 6.44 |

| | | | |
|---|---|---|---|
| CE | 317 | 272, 196 | 9.45 |
| EME | 345 | 317, 182 | 3.77 |
| *Retention Time (RT) varies with column length. Deuterated (D3) internal standards are used throughout. Corresponding ions for internal standards are M+3. Refer to attached method for acquisition parameters (OPICOCSIM.M) | | | |

## 9 INTERPRETATION OF RESULTS

9.1 Assay performance including but not limited to precision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.

9.2 Limits of detection in blood and urine are as follows:

| Drug | LOD and LOQ (Blood) ng/mL | LOD (Urine) ng/mL |
|---|---|---|
| Morphine | 20 | 10 |
| Codeine | 10 | 10 |
| 6-AM (6-acetyl Morphine) | 5 | 10 |
| Cocaine | 5 | 10 |
| Benzoylecgonine | 5 | 10 |
| Cocaethylene | 5 | 10 |
| Ecgonine Methyl Ester | 5 | 20 |

9.3 Qualitative identification is determined using characteristic retention time (RT) and mass spectral characteristics. For Selected Ion Monitoring (SIM), all three characteristic ions must be present. Ion intensity and ratios should be taken into consideration. Ion ratios should be within ± 20% of the calibrator or QC. However, ions of low intensity may not be within this range for all case samples to approval of the technical leader or section manager. If drugs other than the target analytes are suspected, samples may be re-injected using alternative methods of acquisition. If keto-opioids (hydrocodone, oxycodone, hydromorphone, or oxymorphone) are present, they should be re-extracted and analyzed using the appropriate procedure.

9.4 For quantitative analysis, calibrators are typically prepared at concentrations between 20 and 1000 ng/mL. Although the limit of quantitation for some drugs is lower than 20 ng/ml, concentrations in the range between the limit of quantitation and the lowest calibrator may be reported as “less than 20 ng/mL”. See Quantitative Analysis below. An independent control is included in each run.

## 10 QUALITY CONTROLS

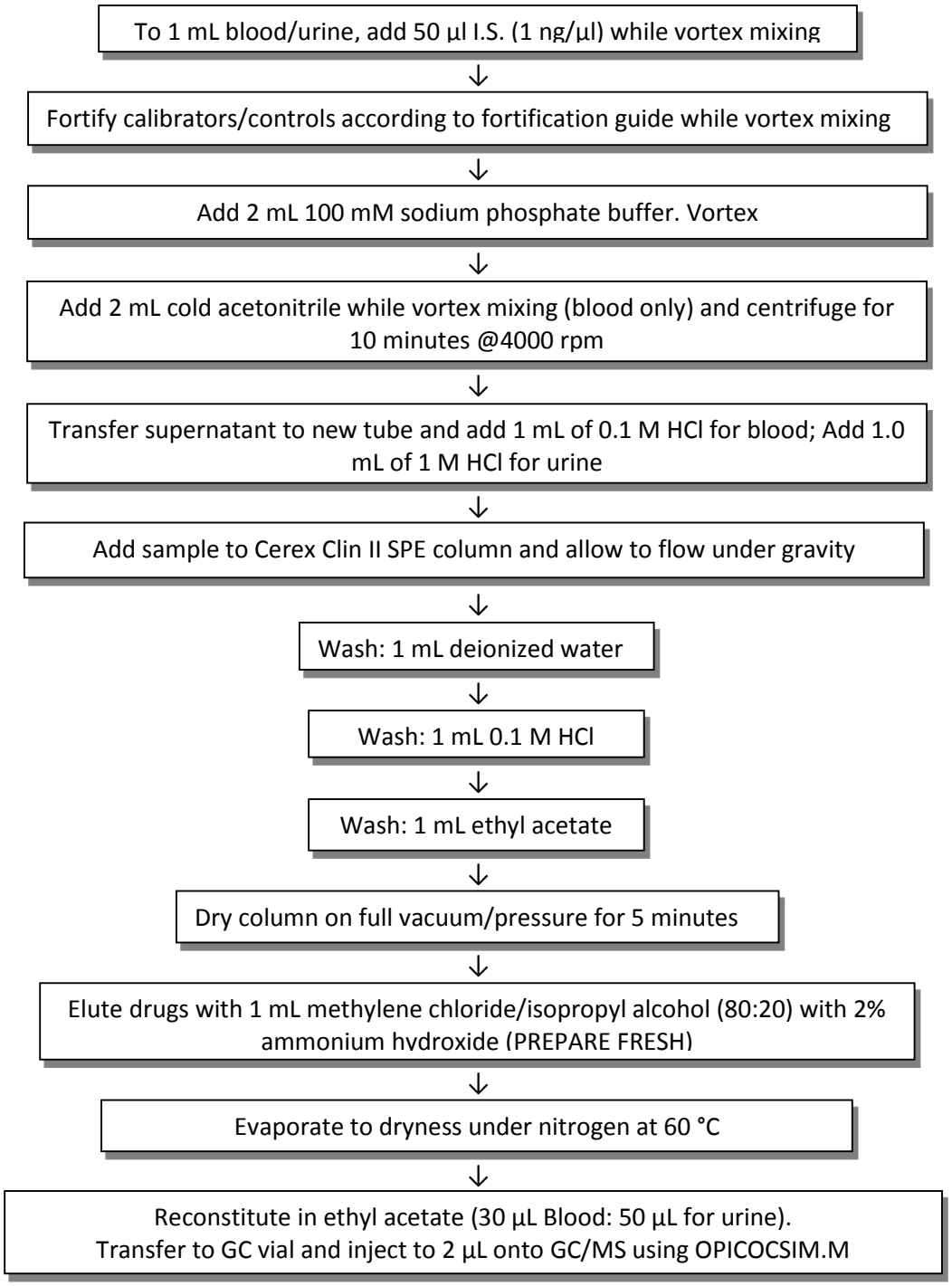
10.1 For quantitative analysis use external QCs (e.g., UTAK QCs) when available. Record the calculated concentration in the appropriate QC Log.

10.1.1 UTAK Drugs of Abuse Level I QC contains 100 ng/mL of morphine, codeine, cocaine, BE and CE.

10.1.2 UTAK Drugs of Abuse Level 2 QC contains 500 ng/mL of morphine, codeine, cocaine, BE and CE.

10.2 If total morphine is to be determined, both the working standard and deuterated internal standard are glucuronidated (morphine-3-glucuronide and morphine-3-glucuronide-d3, respectively).

## 11 FLOWCHART FOR OPIATE/COCAINE ANALYSIS


To 1 mL blood/urine, add 50 µl I.S. (1 ng/µl) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 2 mL 100 mM sodium phosphate buffer. Vortex
Add 2 mL cold acetonitrile while vortex mixing (blood only) and centrifuge for 10 minutes @4000 rpm
Transfer supernatant to new tube and add 1 mL of 0.1 M HCl for blood; Add 1.0 mL of 1 M HCl for urine
Add sample to Cerex Clin II SPE column and allow to flow under gravity
Wash: 1 mL deionized water
Wash: 1 mL 0.1 M HCl
Wash: 1 mL ethyl acetate
Dry column on full vacuum/pressure for 5 minutes
Elute drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH)
Evaporate to dryness under nitrogen at 60 °C
Reconstitute in ethyl acetate (30 µL Blood: 50 µL for urine).
Transfer to GC vial and inject to 2 µL onto GC/MS using OPICOCSIM.M

## 12 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | OPICOC Working Standard Conc. (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 20 | 1 | 20 |
| 1 | 50 | 1 | 50 |
| 1 | 100 | 1 | 100 |
| 1 | 250 | 10 | 25 |
| 1 | 500 | 10 | 50 |
| 1 | 1000 | 10 | 100 |

## 13 LITERATURE AND SUPPORTING DOCUMENTATION

13.1 Kerrigan S; Goldberger DA. Opioids in Principles of Forensic Toxicology, 3rd Edition. Levine B (Ed). AACC Press, Washington DC, 2009, pp 225-244.

13.2 Isenschmid DS. Cocaine in Principles of Forensic Toxicology 3rd Edition. Levine B (Ed). AACC Press, Washington DC, 2009, pp 245-268.

13.3 Grinstead GF. A closer look at acetyl and pentafluoropropionyl derivatives for quantitative analysis of morphine and codeine by gas chromatography/mass spectrometry. J Anal Toxicology 1991 Nov-Dec; Vol. 15(6), pp. 293-8.

13.4 Adejan RE; Schmitt G; Wu M; Meyer C. Determination of cocaine and benzoylecgonine by derivatization with iodomethane-D3 or PFPS/HFIP in human blood and urine using GC/MS (EI or PCI mode). J Anal Toxicology 1993 Jan-Feb; Vol 17(1), pp. 51-5.

13.5 Method File: OPICOC.M

# Phencyclidine Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

This document outlines the confirmatory procedure for quantitative blood and qualitative urine analysis of Phencyclidine (PCP) by Solid Phase Extraction (SPE) and Gas Chromatography/Mass Spectrometry (GC/MS). Drugs are isolated from the matrix using basic extraction. Deuterated Internal Standard and Selective Ion Monitoring (SIM) are used in Electron Ionization mode.

## 2 SCOPE

This procedure is used for confirmatory analysis of PCP in blood and urine.

## 3 SAFETY

This procedure must be conducted in accordance with the HFSC Health and Safety Manual and Quality Manual. All case specimens should be treated with Universal Blood borne Pathogen Precautions. Appropriate personal protective equipment must be worn during sample and reagent preparation and when handling caustic chemicals. Flammable liquids and vapors may cause eye, skin and respiratory tract irritation. Derivatization reagents are toxic and must be handled in a chemical safety hood or well ventilated area. Material Safety Data Sheets (MSDS) are available in the laboratory.

## 4 REAGENTS

Refer to SOP: Reagents for Drug Toxicology for preparation information

4.1 1M Glacial Acetic Acid
4.1 100 mM, pH 6.0 Phosphate buffer
4.2 80:20 Methylene Chloride/Isopropanol
4.3 Concentrated Ammonium Hydroxide
4.4 Deionized (DI) Water [7732-18-5]
4.5 Ethyl Acetate
4.6 Hexane
4.7 Methanol
4.8 Blank Blood (Potassium Oxalate and Sodium Fluoride preserved)
4.9 Blank Urine (No preservatives added)

## 5 EQUIPMENT

5.1 Instrumentation using a method that is approved and validated for use in the section
5.1.1 Air Displacement Pipettes (100-1000 µL; 20-200 µL; 2-20 µL)
5.1.2 Cerex PolyChrom Clin II SPE columns
5.1.3 Positive Pressure manifold or equivalent
5.1.4 Analytical Balance
5.1.5 pH Meter
5.1.6 Evaporator
5.1.7 Vortex mixer
5.1.8 Centrifuge
5.1.9 Agilent 7890 GC 5975 MSD

5.1.10 Round bottom tubes, conical tubes, GCMS vials and caps.

## 6 INSTRUMENT PARAMETERS

6.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.3 mL/min with an injection volume of 2 µL in split mode (10:1)

6.2 GC/MS: Agilent 7890

| | |
|---|---|
| Initial Temperature: | 140°C<br>Hold for 0.5 min<br>30°C/min to 290°C<br>Hold for 2.5 min<br>50°C/min to 310°C<br>Hold for 2.6 min |
| Total Run Time: | 11 min |
| Injector Temperature: | 250°C |
| Interface Temperature: | 280°C |
| MS Quads: | 150°C |
| MS Source: | 230°C |

6.3 Wash solvents for autosampler: METHANOL and ETHYL ACETATE are used as the wash solvents. A minimum of 6 pre and 6 post injection rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 7 MAINTENANCE

For detailed instructions on the operation of the GC/MS refer to TX-07-07 Maintenance and Use of the HP 5975 MSD.

## 8 CALIBRATION

Calibrators and controls are prepared using drug-free urine or drug-free blood. Preparation of stock solutions and working standards are documented in a retrievable format.

### 8.1 Calibrator Solutions

#### 8.1.1 Stock Solution (0.1 mg/mL)

Add 1000 µL of Phencyclidine (1.0 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol.

**Storage**: Store refrigerated. **Discard**: 6 months.

#### 8.1.2 Working solution A (1 ng/µL)

Add 100 µL of 0.1 mg/mL stock solution to a 10 mL volumetric flask and bring to volume with methanol.

**Storage**: Store refrigerated. **Discard**: 3 months.

#### 8.1.3 Working Solution B (0.1 ng/µL)

Add 1000 µL of 1 ng/µL working solution A to a 10 mL volumetric flask and bring to volume with methanol.

**Storage**: Store refrigerated. **Discard**: 3 months.

9 **INTERNAL STANDARD**

This solution consists of Phencyclidine-D5.

9.1 **I.S. Working Solution (1 ng/µL)**

Add 100 µL of the deuterated drug standard (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol.

**Storage**: Store refrigerated. **Discard**: 6 months.

10 **Quality Control Solutions**

**NOTE:** Calibrators and controls must be prepared from separate lots or sources of certified reference material. Controls may be purchased or prepared in-house. In-house prepared controls shall be verified according to SOP: Preparation and Validation of Drug Standards, Spiking Solutions, Controls and Reagents.

10.1 **QC Stock Solution (10 ng/µL)**

Transfer 250 µL of PCP reference material (1000 ng/µL) into a 25 mL volumetric flask containing approximately 15 mL of methanol. Bring to volume with methanol.

**Storage**: Store frozen in amber vial. **Discard**: 12 months.

10.2 **PCP HIGH Blood QC Samples (80 ng/mL)**

Transfer 750 µL of PCP Stock QC solution (10 ng/µL) into 100 mL volumetric flask containing approximately 50 mL of room temperature blank blood. Bring to volume with blank blood. Transfer 1.2 mL aliquots to appropriately labeled 2.0 mL snap-cap conical tubes. Assign lot number and expiration date.

**Storage**: Store frozen. **Discard**: 6 months.

10.3 **PCP LOW BLOOD QC Samples (15 ng/mL)**

Transfer 150 µL of PCP Stock QC solution (10 ng/µL) into 100 mL volumetric flask containing approximately 50 mL of room temperature blank blood. Bring it to volume with blank blood. Transfer 1.2 aliquots to appropriately labeled 2.0 mL snap-cap conical tubes. Assign lot number and expiration date.

**Storage**: Store frozen. **Discard**: 6 months.

10.4 **PCP Negative Urine QC Samples (10 ng/mL)**

Transfer 50 µL of PCP Stock QC solution (10 ng/µL) into 100 mL volumetric flask containing approximately 50 mL of room temperature blank urine. Bring it to volume with blank urine. Transfer 1.2 aliquots to appropriately labeled 2.0 mL snap-cap conical tubes. Assign lot number and expiration date.

**Storage**: Store frozen. **Discard**: 6 months.

10.5 **PCP Positive Urine QC Samples (20 ng/mL)**

Transfer 200 µL of PCP Stock QC solution (10 ng/µL) into 100 mL volumetric flask containing approximately 50 mL of room temperature blank urine. Bring it to volume with blank urine. Transfer 1.2 aliquots to appropriately labeled 2.0 mL snap-cap conical tubes. Assign lot number and expiration date.

**Storage**: Store frozen. **Discard**: 6 months.

10.6 **Dilution Control**

A 2x dilution of High Blood QC is analyzed in the batch, if necessary, to verify dilution results of case specimens.

## 11 SAMPLE PREPARATION

11.1 Aliquot 1 mL blank blood, controls and case samples into labeled round bottom glass culture tube. NOTE: Dilution of a specimen may be necessary for samples that contain elevated concentration of drug. Any changes in sample volume that deviate from the technical procedure must be documented in the case folder.

11.2 Fortify calibrators according to the table below:

| Volume of Blood (mL) | Phencyclidine Target Concentration (ng/mL) | Phencyclidine Working Standard Concentration (ng/µL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 5 | B (0.1) | 50 |
| 1 | 10 | B | 100 |
| 1 | 25 | A (1) | 25 |
| 1 | 50 | A | 50 |
| 1 | 100 | A | 100 |

11.3 Add 50 µL of Internal Standard Solution (1 ng/µL) while vortex mixing. Final concentration of internal standard in each sample is 50 ng/mL.

11.4 Add 2 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex. Centrifuge at 4000 RPM for 10 minutes.

11.5 Place collection tubes or reservoir (for waste) into vacuum or positive pressure manifold rack.

11.6 Add the sample to Polychrom Clin II Column. Draw the sample through using sufficient vacuum/pressure when necessary.

11.7 Wash column with 1 mL of deionized water.

11.8 Wash column with 1 mL of 1M acetic acid.

11.9 Dry column on full vacuum/pressure for 5 minutes.

11.10 Wash column with 1 mL of hexane.

11.11 Wash column with 1 mL ethyl acetate.

11.12 Wash column with 1 mL methanol.

11.13 Dry column on full vacuum/pressure for 5 minutes.

11.14 Replace waste tubes/reservoir with labelled conical collection tubes.

11.15 Elute Basic Drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH).

Preparation: 2% ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol. For instance, in a 25 mL volumetric flask, add 0.5 mL concentrated ammonium hydroxide. Bring to volume with 80:20 methylene chloride/isopropanol.

11.16 Turn on vacuum/pressure for a few seconds to ensure all elution solvent has drained from the column.

11.17 Remove tubes from rack and evaporate to dryness (approximately 10 minutes) under nitrogen at 50°C-60°C.

11.18 Reconstitute in 30 µL of ethyl acetate (or an appropriate volume), vortex, and transfer to autosampler vials.

11.19 Inject 2 µL onto the GC/MS using PCP.M method and SIM acquisition.

12 **SEQUENCE TABLE (Example only)**

Note: A minimum of 2 low and 2 high controls must be included in every run that contains blood; a minimum of 2 positive and 2 negative controls must be included in every run that contains urine**.**

| Blood |
|---|
| Calibrator 1 |
| Calibrator 2 |
| Calibrator 3 |
| Calibrator 4 |
| Calibrator 5 |
| Negative |
| High QC |
| Low QC |
| 10 Case Samples |
| Low QC |
| 10 Case Samples e.g.: 1,2,3,4,5 |
| High QC |
| 10 Case Samples |
| High QC |
| Low QC |

| Urine |
|---|
| Cutoff Calibrator |
| Negative |
| Positive QC |
| Negative QC |
| 10 Case Samples |
| Negative QC |
| 10 Case Samples e.g.: 1,2,3,4,5 |
| Positive QC |
| 10 Case Samples |
| Positive QC |
| Negative QC |

13 **METHOD OF CALCULATION**

See SOP: In-Process Calibration and Quality Control for Toxicology Testing for general information on calibration and control of assays.

| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| PCP | 242 | 243, 200 | 4.70 |
| PCP-d5 | 205 | 190, 247 | 4.69 |

*Retention Time (RT) varies with column length. Deuterated (D5) internal standards are used throughout. Refer to the method for acquisition parameters of PCP.M

13.1 **Blood**

13.2 The Limit of Detection (LOD) and Limit of Quantitation (LOQ) in blood are 5 ng/ml.

13.2.1 Analyte concentrations in blood are determined by linear regression (y = mx + b) with 1/x weighting, based on the ratio of the peak area of the calibrator divided by the peak area of the internal standard. The calibration curve is calculated by instrument software.

13.2.2 All calibrators must back calculate to within ±20% of target

13.2.3 A maximum of 1 calibrator may be dropped from the curve. If a calibrator is not used this must be documented on the Calibration Curve along with an explanation as to why it was not included.

13.3 **Urine**

13.3.1 The Limit of Detection (LOD) and Limit of Quantitation (LOQ) in blood are10 ng/ml.

13.3.2 Urine is calibrated using a 1 point calibration curve

14 **QUALITY CONTROL ACCEPTANCE CRITERIA**

14.1 All Blood QC samples must quantify within ±20% of target.

14.2 All urine controls must give the expected result (negative must be negative, positive must be positive).

14.3 All blood QCs must pass to report blood results.

14.4 All urine QCs must pass to report urine results.

15 **REPORTING RESULTS**

15.1 **Blood**

15.1.1 All ion ratios must be within ±20% of the average ion ratio of the calibrators.

15.1.2 Quantitative results are reported by rounding to the ones place (ex. 5.6 ng/mL reported as 6 ng/mL; 9.2 ng/mL reported as 9 ng/mL; 10.7 ng/mL reported as 11 ng/mL).

15.2 **Urine**

15.2.1 All ion ratios must be within ±20% of the ion ratio of the cutoff calibrators.

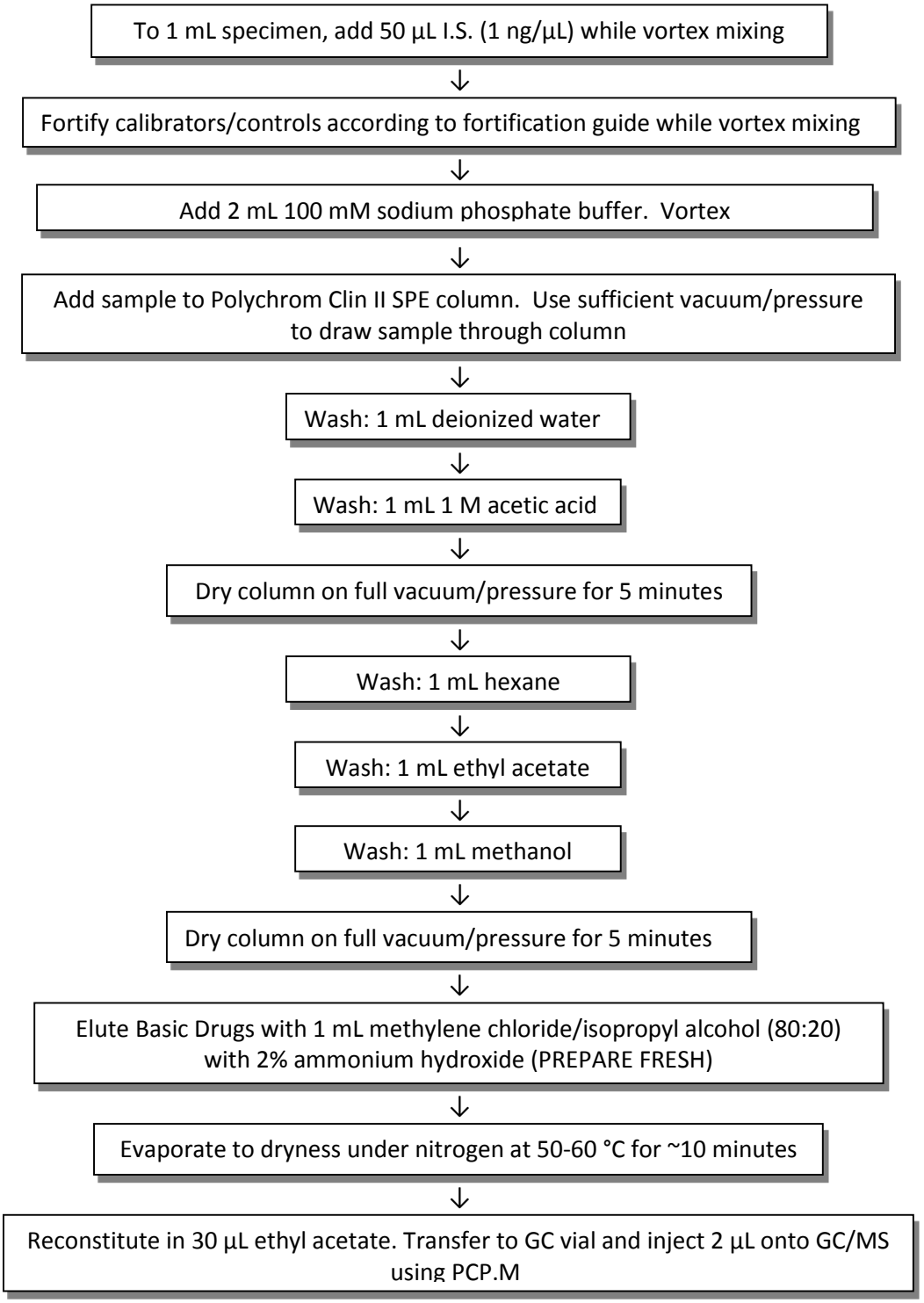
15.2.2 If the response ratio of the target ion for PCP is greater than the response ratio of the 10 ng/mL cutoff calibrator the result is reported as “Positive”.

15.2.3 If the response ratio of the target ion for PCP is less than the response ratio of the 10 ng/mL cutoff calibrator the result is reported as “None Detected”.

16 **LIMITATIONS**

Validation was performed using human blood without preservatives for the preparation of calibrators and controls. Evaluation of samples collected in gray top tubes indicates that one of the additives in these tubes causes an elevated m/z 200 ion. As a result, the qualifier ion ratio in samples containing <10 ng/mL does not fall within +/- 20% of the average calibrator ion ratio. The quantitative results are not significantly affected.

17 **Flow Chart for PCP Preparation**


To 1 mL specimen, add 50 µL I.S. (1 ng/µL) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 2 mL 100 mM sodium phosphate buffer. Vortex
Add sample to Polychrom Clin II SPE column. Use sufficient vacuum/pressure to draw sample through column
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes
Wash: 1 mL hexane
Wash: 1 mL ethyl acetate
Wash: 1 mL methanol
Dry column on full vacuum/pressure for 5 minutes
Elute Basic Drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH)
Evaporate to dryness under nitrogen at 50-60 °C for ~10 minutes
Reconstitute in 30 µL ethyl acetate. Transfer to GC vial and inject 2 µL onto GC/MS using PCP.M

## 18 LITERATURE AND SUPPORTING DOCUMENTATION

18.1 Kunsman, G.W., et al. Phencyclidine Blood Concentrations in DRE Cases. J Anal Toxicol 1997 October; Vol. 21, pp. 498-502.

18.2 Grieshaber, A., et al. Stability of Phencyclidine in Stored Blood Samples. J Anal Toxicol 1998 October; Vol. 22, pp. 515-519.

18.3 Pestaner, J.P., et al. Sudden Death During Arrest and Phencyclidine Intoxication. American J Forensic Medicine and Pathology. 2003. June. Vol. 24. No. 2, pp 119-122.

18.4 R.C. Baselt. Phencyclidine. Disposition of Toxic Drugs and Chemicals in Man. 8th edition. Biomedical Publications, Foster City, CA, pp 1223-1226.

18.5 Method file: PCP.M

# Stimulants Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for methamphetamine, amphetamine, ephedrine, pseudoephedrine, 3,4-methylenedioxymethamphetamine (MDMA), methylenedioxy-amphetamine (MDA), and 3,4-methylenedioxy-*N*-ethylamphetamine(MDEA). Drugs are isolated from the matrix using solid phase extraction (SPE). Perfluoroacyl derivatives are prepared and analyzed using gas chromatography/mass spectrometry (GC/MS), deuterated internal standards, and selective ion monitoring (SIM) in electron ionization (EI) mode.

## 2 SCOPE

Confirmatory analysis of target analytes from toxicology specimens including but not limited to blood and urine. Urine confirmations are reported only qualitatively.

## 3 REAGENTS AND STANDARDS

3.1 Stimulant Working Standards: Two working standards are used. Each contains methamphetamine, amphetamine, ephedrine, pseudoephedrine, MDEA, MDA, and MDMA at either 10 ng/µL or 1 ng/µL, respectively.

3.1.1 **Preparation of 10 ng/µL Stimulant Working Standard**: Add 1000 µL of each drug standard (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.1.2 **Preparation of 1 ng/µL Stimulant Working Standard**: Add 1000 µL of the 10 ng/µL Stimulant Working Standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2 Internal Standard Solution: This solution consists of methamphetamine-d14, amphetamine-d11, ephedrine-d3, pseudoephedrine-d3, MDEA-d5, MDA-d5, and MDMA-d5 in methanol at 10 ng/µL.

3.2.1 Preparation of (10 ng/µL) Internal Standard Solution: Add 1000 µL of each deuterated drug standard (0.1 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.3 1M Acetic Acid

3.4 Acidic Methanol (1% HCl in methanol)

3.5 Phosphate Buffer pH 6.0

3.6 Elution Solvent: 2% ammonium hydroxide (concentrated) in ethyl acetate plus 1 mL of methanol

3.7 PFPA (pentafluoropropionic anhydride) for derivatization. Store at room temperature. Discard if there is a change in color.

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)

4.2 Cerex PolyChrom Clin II SPE columns

4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold

4.4 Pan balance

4.5 pH meter
4.6 Evaporator
4.7 Vortex mixer
4.8 Centrifuge
4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD
4.14 Stir plate and stir bars

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.1 mL/min with an injection volume of 2 µL in split mode (10:1).

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 80 °C<br>Hold for 0.5 minutes<br>30 °C/min to 290 °C<br>Hold for 5 minutes |
| Total Run Time: | 12.5 minutes |
| Injector Temperature: | 260 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

6.1 Sonication (15 minutes) and centrifugation (15 minutes at 4,000 rpm) is permitted for biological samples such as blood, tissue homogenates, urine, or controls as needed.

6.2 **BLOOD:**

6.2.1 In a round bottom glass centrifuge tube, add 1 mL blood and 25 µL Internal Standard Solution (10 ng/µL) while vortex mixing. Final concentration of internal standard in each sample is 250 ng/mL. NOTE IF THE SAMPLE IS KNOWN TO CONTAIN A HIGH CONCENTRATION OF DRUG, ALIQUOT A SMALL VOLUME, ADD BUFFER AS THE DILUENT AND DOCUMENT IN THE CASE FOLDER.

6.2.2 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. The typical calibration range for quantitative analysis is 20 -1000 ng/mL. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. An in-house control

containing 100 ng/mL of each analyte is recommended. Use appropriate external controls (e.g., UTAK Whole Blood Controls) when available.

6.2.3 Add 2 mL of phosphate buffer, pH 6.0. Vortex.

6.2.4 Centrifuge samples at 4,000 rpm for at least 10 minutes.

6.2.5 Place collection tubes or reservoir (for biological waste) into vacuum manifold rack.

6.2.6 Pour sample onto SPE columns. If sample quality is poor, decant the supernatant layer into a clean tube and avoid putting solid material onto the column.

6.2.7 Use sufficient vacuum to draw blood samples through the column (approximately 5 psi.).

6.2.8 Wash column with 1 mL deionized water.

6.2.9 Remove collection tubes or reservoir (for biological waste) and replace with collection tubes or reservoir (for organic waste). Dispose of biological waste.

6.2.10 Wash column with 1 mL 1 M acetic acid

6.2.11 Dry column on full vacuum/pressure for 5 minutes.

6.2.12 Wash column with 1 mL hexane.

6.2.13 Wash column with 1 mL ethyl acetate.

6.2.14 Wash column with 1 mL methanol.

6.2.15 Place conical collection tubes into the rack. If using Teflon inserts and they appear dirty, replace them.

6.2.16 Elute drugs with 1 mL elution solvent (2% ammonium hydroxide in ethyl acetate plus 1 mL methanol).

6.2.17 Turn on vacuum/pressure for a few seconds to ensure all elution solvent has drained from the column.

6.2.18 Remove tubes from rack and add 30 µL of acidic methanol.

6.2.19 Evaporate to dryness under nitrogen at a temperature not to exceed 50° C, avoid over drying samples.

6.2.20 IN THE FUME HOOD, add 50 µL ethyl acetate and 50 µL PFPA. Cap tightly. Vortex. Heat at 70° C for 30 minutes. Allow samples to come to room temperature.

6.2.21 Evaporate to dryness under nitrogen at a temperature not to exceed 50° C.

6.2.22 Reconstitute in 20 µL ethyl acetate (or appropriate volume), vortex, and transfer to autosampler vials.

6.2.23 Inject 2 µL onto the GC/MS using STIMSIM.M

6.3 **URINE:**

6.3.1 In a round bottom centrifuge tube, add 1 mL urine and 25 µL Internal Standard Solution (10 ng/µL) while vortex mixing. The total concentration of internal standard is 250 ng/mL.

6.3.2 For qualitative analysis include a minimum of one negative and one positive control in each run. For preparation of a 200 ng/mL positive control, add 20 µL of Stimulant Working Standard Solution (10 ng/µL) to 1 mL urine while vortex mixing.

6.3.3 Add 2 mL phosphate buffer (pH 6.0). Vortex.

6.3.4 If sample is cloudy or contains particulates, centrifuge for 15 minutes, 4000 rpm.

6.3.5 Proceed from step 4 through step 20 of blood procedure.

6.3.6 Evaporate to dryness under nitrogen at a temperature not to exceed 50° C. Reconstitute in 40 µL ethyl acetate (or appropriate volume), vortex, and transfer to autosampler vials.

Samples suspected of containing high concentrations of drug may be reconstituted in a larger volume of ethyl acetate if necessary. Presumptive immunoassay test results can be used as a guide for reconstitution volume for urine samples.

6.3.7 Inject 2 µL onto the GC/MS using STIMSIM.M.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.

7.2 SIM acquisition: STIMSIM.M. Use this method to perform targeted analysis for amphetamine, methamphetamine, ephedrine, pseudoephedrine, MDMA, MDA, and MDEA.

| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Amphetamine | 190 | 118, 91 | 4.172 |
| Methamphetamine | 204 | 160, 118 | 4.676 |
| Ephedrine | 204 | 160,294 | 4.610 |
| Pseudoephedrine | 204 | 160, 294 | 4.854 |
| MDMA | 204 | 339, 162 | 6.022 |
| MDA | 325 | 162, 135 | 5.565 |
| MDEA | 353 | 218, 190 | 6.164 |

| Drug Internal Standard | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Amphetamine-D11 | 194 | 128,98 | 4.153 |
| Methamphetamine-D14 | 211 | 163, 128 | 4.643 |
| Ephedrine-D3 | 207 | 163,297 | 4.599 |
| Pseudoephedrine-D3 | 207 | 163, 297 | 4.843 |
| MDMA-D5 | 208 | 344, 163 | 6.009 |
| MDA-D5 | 330 | 167, 136 | 5.554 |
| MDEA-D5 | 358 | 223, 191 | 6.144 |

*Retention Time (RT) varies with column length. Deuterated internal standards are used throughout.

## 8 INTERPRETATION OF RESULTS

8.1 Assay performance including but not limited to precision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.

8.2 Limits of detection in blood and urine are as follows:

| Drug | LOD (Blood) ng/mL | LOQ (Blood) ng/mL | LOD (Urine) ng/mL |
|---|---|---|---|
| Amphetamine | 10 | 10 | 10 |
| Methamphetamine | 10 | 10 | 10 |
| Ephedrine | 10 | 10 | 10 |
| Pseudoephedrine | 10 | 10 | 10 |
| MDMA | 20 | 20 | 25 |
| MDA | 20 | 20 | 25 |
| MDEA | 20 | 20 | 25 |

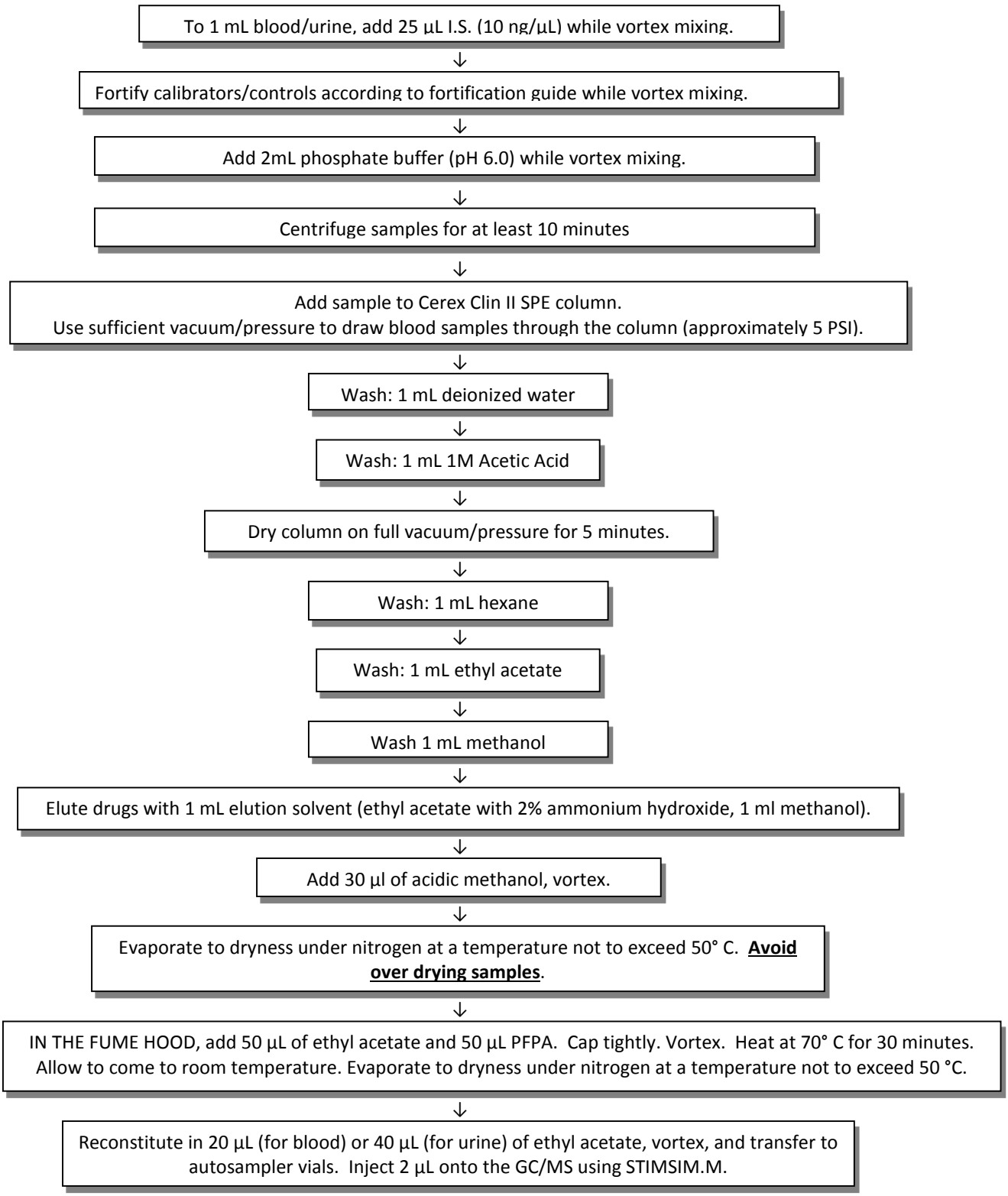
8.3 MDEA is reported qualitative only. For MDA and MDMA curve is loaded till 250 ng/ml as higher concentration causes interferences in the Internal Standard. IF the sample contains greater than 500ng/ml of MDA and MDMA then dilution is needed.

## 9 QUALITY CONTROLS

9.1 Positive and negative QCs must be included in each run. For qualitative analysis, each batch must contain a blank (drug-free) extract and an extracted drug standard.

9.2 For quantitative analysis, use additional external QCs (e.g., UTAK QCs) when available. UTAK Drugs of Abuse Level I QC contains 100 ng/mL of amphetamine, methamphetamine, MDA, MDEA, and MDMA. Record the calculated concentrations in the appropriate QC Log.

## 10 FLOWCHART FOR STIMULANT ANALYSIS


To 1 mL blood/urine, add 25 µL I.S. (10 ng/µL) while vortex mixing.
Fortify calibrators/controls according to fortification guide while vortex mixing.
Add 2mL phosphate buffer (pH 6.0) while vortex mixing.
Centrifuge samples for at least 10 minutes
Add sample to Cerex Clin II SPE column.
Use sufficient vacuum/pressure to draw blood samples through the column (approximately 5 PSI).
Wash: 1 mL deionized water
Wash: 1 mL 1M Acetic Acid
Dry column on full vacuum/pressure for 5 minutes.
Wash: 1 mL hexane
Wash: 1 mL ethyl acetate
Wash 1 mL methanol
Elute drugs with 1 mL elution solvent (ethyl acetate with 2% ammonium hydroxide, 1 ml methanol).
Add 30 µl of acidic methanol, vortex.
Evaporate to dryness under nitrogen at a temperature not to exceed 50° C. Avoid over drying samples.
IN THE FUME HOOD, add 50 µL of ethyl acetate and 50 µL PFPA. Cap tightly. Vortex. Heat at 70° C for 30 minutes. Allow to come to room temperature. Evaporate to dryness under nitrogen at a temperature not to exceed 50 °C.
Reconstitute in 20 µL (for blood) or 40 µL (for urine) of ethyl acetate, vortex, and transfer to autosampler vials. Inject 2 µL onto the GC/MS using STIMSIM.M.

## 11 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | Stimulant Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 20 | 1 | 20 |
| 1 | 50 | 1 | 50 |
| 1 | 100 | 1 | 100 |
| 1 | 250 | 10 | 25 |
| 1 | 500 | 10 | 50 |
| 1 | 1000 | 10 | 100 |

## 12 LITERATURE AND SUPPORTING DOCUMENTATION

12.1 Villamor JL; Bermejo AM; Fernández P; Tabernero MJ. A new GC-MS method for the determination of five amphetamines in human hair. J Anal Toxicol 2005 March; Vol. 29(2), pp.135-139.

12.2 Churley M; Robandt PV; Kuhnle JA; Lyons TP; Bruins MR. Extraction of amphetamine and methamphetamine from urine specimens with Cerex Polycrom Clin II Solid Phase Extraction columns and the Speedisk 48 Pressure Processor. J Anal Toxicol 2002 September; Vol 26(6), pp.347-354.

12.3 Valtier S; Cody JT. Evaluation of internal standards for the analysis of amphetamine and methamphetamine. J Anal Toxicol 1995 October; Vol. 19 (6), pp. 375-380.

12.4 Awad T; DeRuiter J; Clark CR. GC-MS Analysis of Acylated Derivatives of the side chain and ring regioisomers of methylenedioxymethamphetamine. J of Chromatographic Science 2005 July; Vol. 43 (6), pp. 296-303.

12.5 Baselt RC. Disposition of Toxic Drugs and Chemicals in Man, 8th Edition. Biomedical Publications, Foster City, CA, 2008.

12.6 Method file: STIMSIM.

# Zolpidem Confirmation by Gas Chromatography/Mass Spectrometry (GC/MS)

## 1 PURPOSE

A targeted analysis is performed for confirmatory analysis of zolpidem by solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS). Drugs are isolated from the matrix using a basic extraction. Zolpidem $D_7$ is used as the internal standard and selective ion monitoring (SIM) is used in electron ionization (EI) mode.

## 2 SCOPE

Confirmatory analysis of zolpidem from toxicology specimens including but not limited to blood. Urine confirmations are reported only qualitatively and are typically reported using Basic, Acidic and Neutral Drugs Confirmation by GC/MS.

## 3 REAGENTS AND STANDARDS

3.1 Zolpidem Working Standard

3.1.1 Preparation of 10 ng/µL Zolpidem Working Standard: Add 100 µL of zolpidem (1.0 mg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.1.2 Preparation of 1 ng/µL Zolpidem Working Standard: Add 1 mL of 10 ng/µL working standard to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (3 month expiration).

3.2 Internal Standard Solution

3.2.1 Preparation of 10 ng/µL Internal Standard Stock Solution: Add 1 mL of Zolpidem $–D_7$ (100 µg/mL) to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.2.2 Preparation of 1 ng/µL Working Internal Standard: Add 1 mL of 10 ng/µL Zolpidem $–D_7$ to a 10 mL volumetric flask and bring to volume with methanol. Store refrigerated (6 month expiration).

3.3 1M Acetic Acid

3.4 100 mM, pH 6.0 Phosphate Buffer

3.5 Elution Solvent (2% ammonium hydroxide concentrated in 80:20 methylene chloride/isopropanol)

3.6 Methylene Chloride/Isopropanol (80:20)

## 4 EQUIPMENT AND MATERIALS

4.1 Air displacement pipettes (100-1000 µL; 20-200 µL; 2-20 µL)

4.2 Cerex PolyChrom Clin II SPE columns

4.3 Vacuum manifold with disposable Teflon inserts or positive pressure SPE manifold

4.4 Pan balance

4.5 pH meter

4.6 Evaporator

4.7 Vortex mixer

4.8 Centrifuge

4.9 Tissue homogenizer
4.10 Sonicator
4.11 Heating block
4.12 Vacuum pump or house vacuum
4.13 Agilent 7890 GC 5975 MSD

## 5 INSTRUMENTAL PARAMETERS

5.1 Capillary Column: 30 m HP-5MS Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 1.2 mL/min with an injection volume of 2 µL in split mode (10:1).

5.2 GC/MS: Agilent 7890A

| | |
|---|---|
| Initial Temperature: | 140 °C<br>Hold for 0.5 minutes<br>30 °C/min to 290 °C<br>Hold for 2.5 minutes<br>50 °C/min to 310 °C<br>Hold for 2.6 minutes |
| Total Run Time: | 11 minutes |
| Injector Temperature: | 250 °C |
| Interface Temperature: | 280 °C |
| MS Quads: | 150 °C |
| MS Source: | 230 °C |

5.3 Wash solvents for autosampler: Methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

## 6 EXTRACTION PROCEDURE

6.1 Blood, Urine, and all Other matrices

6.1.1 In a round bottom glass culture tube, add 1 mL of specimen and 100 µL WORKING Internal Standard Solution (1ng/uL) while vortex mixing (total concentration is 100 ng/mL).

6.1.2 **Dilutions:** If the sample is known to contain a high concentration of drug, it should be diluted prior to analysis. Aliquot the desired volume of blood and make up to volume using phosphate buffer. DOCUMENT THESE CHANGES IN THE CASE RECORD. Then proceed from step 4 of the blood procedure.

6.1.3 Fortify all controls and calibrators while vortex mixing using the appropriate amount of working standard. Refer to the fortification guide for guidance on preparation of calibrators. CONTROLS AND CALIBRATORS MUST COME FROM INDEPENDENT STOCK SOLUTIONS. A minimum of one in-house control should be included in each run. Use appropriate external controls (e.g., UTAK Whole Blood Controls) when available. An in-house quantitative QC of 50 ng/mL is recommended.

6.1.4 Add 2 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex.

6.1.5 Centrifuge all samples for at least 10 minutes at 4000 rpm.

6.1.6 Place collection tubes or reservoir (for waste) into vacuum manifold rack or positive pressure apparatus.

6.1.7 Pour sample onto Polychrom Clin II columns. Use sufficient vacuum/pressure to draw samples through the columns.

6.1.8 Wash column with 1 mL deionized water.

6.1.9 Remove waste tubes/reservoir and replace with fresh waste tubes/reservoir.

6.1.10 Wash column with 1 mL of 1 M acetic acid.

6.1.11 Dry column on full vacuum/pressure for 5 minutes.

6.1.12 Wash column with 1 mL hexane.

6.1.13 Wash column with 1 mL ethyl acetate.

6.1.14 Wash column with 1 mL methanol.

6.1.15 Dry column on full vacuum/pressure for 5 minutes.

6.1.16 Replace waste tubes/reservoir with conical collection tubes. If the Teflon inserts (vacuum manifold only) appear dirty, replace them.

6.1.17 Elute basic drugs with 1mL of elution solvent (2% ammonium hydroxide in methylene chloride/isopropyl alcohol 80:20). ELUTION SOLVENT MUST BE MADE FRESH.

6.1.18 Turn on vacuum or apply positive pressure for a few seconds to ensure all elution solvent has drained from the column.

6.1.19 Evaporate to dryness under nitrogen for 15 minutes at 50-60°C. Reconstitute in 30 µL ethyl acetate (or an appropriate volume), vortex, and transfer to autosampler vials.

6.1.20 Inject 2 µL onto the GC/MS using ZOLPIDEM.

## 7 INSTRUMENTAL ANALYSIS

7.1 Ensure that the daily QC and tune verification have been completed.

7.2 SIM acquisition: ZOLPIDEM.M. Use this method to perform targeted analysis for zolpidem.

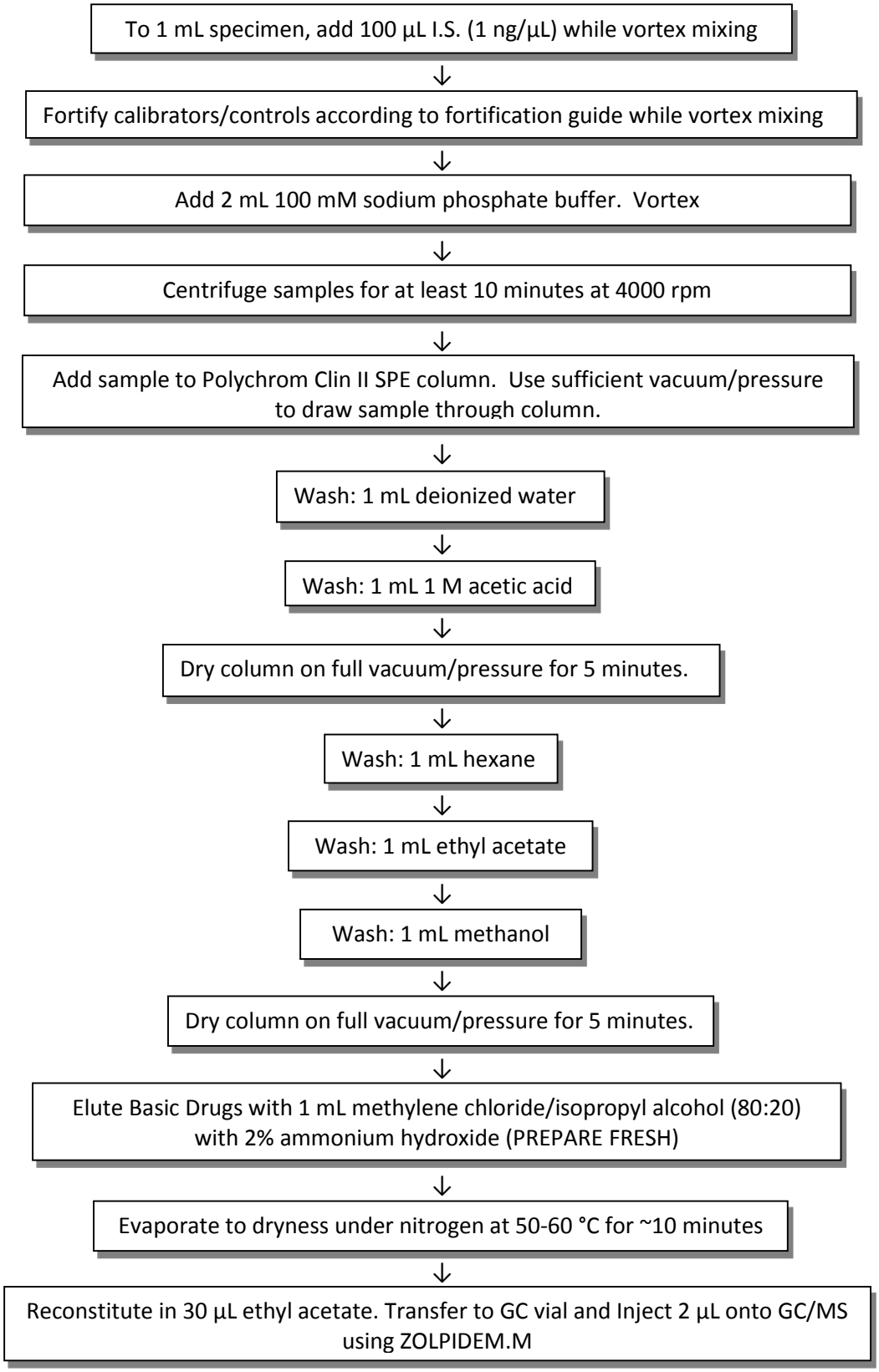
| Drug | Quant Ion | Qualifier Ions | RT* |
|---|---|---|---|
| Zolpidem-D7 | 242 | 314, 223 | 8.05 |
| Zolpidem | 235 | 307, 219 | 8.27 |
| *Retention Time (RT) varies with column length. Deuterated (D7) internal standards are used throughout. Corresponding ions for internal standards are M+7. | | | |

## 8 INTERPRETATION OF RESULTS

8.1 Assay performance including but not limited to precision, accuracy, limit of detection, limit of quantitation, and linearity are summarized in the validation documentation.

8.2 Limit of detection and limit of quantitation of zolpidem in whole blood are 20 ng/mL and 25 ng/mL, respectively.

## 9 FLOWCHART FOR ZOLPIDEM ANALYSIS


To 1 mL specimen, add 100 µL I.S. (1 ng/µL) while vortex mixing
Fortify calibrators/controls according to fortification guide while vortex mixing
Add 2 mL 100 mM sodium phosphate buffer. Vortex
Centrifuge samples for at least 10 minutes at 4000 rpm
Add sample to Polychrom Clin II SPE column. Use sufficient vacuum/pressure to draw sample through column.
Wash: 1 mL deionized water
Wash: 1 mL 1 M acetic acid
Dry column on full vacuum/pressure for 5 minutes.
Wash: 1 mL hexane
Wash: 1 mL ethyl acetate
Wash: 1 mL methanol
Dry column on full vacuum/pressure for 5 minutes.
Elute Basic Drugs with 1 mL methylene chloride/isopropyl alcohol (80:20) with 2% ammonium hydroxide (PREPARE FRESH)
Evaporate to dryness under nitrogen at 50-60 °C for ~10 minutes
Reconstitute in 30 µL ethyl acetate. Transfer to GC vial and Inject 2 µL onto GC/MS using ZOLPIDEM.M

## 10 FORTIFICATION GUIDE

| Volume of Blood (mL) | Target Concentration (ng/mL) | Working Standard Concentration (ng/µL = µg/mL) | Volume Added (µL) |
|---|---|---|---|
| 1 | 20 | 1 | 20 |
| 1 | 50 | 1 | 50 |
| 1 | 100 | 1 | 100 |
| 1 | 250 | 10 | 25 |
| 1 | 500 | 10 | 50 |

## 11 LITERATURE AND SUPPORTING DOCUMENTATION

11.1 Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 7th Ed., Biomedical Publications, Foster City, CA, p 1212-1214 (2004).

11.2 Timothy P. Rohrig and Christine M. Moore. Zolpidem (Review). Forensic Science, Medicine, and Pathology. Human Press (2005).

11.3 Method file: ZOLPIDEM.M

# Analysis of Alcohol and Other Volatiles by Headspace GC/FID

## 1 PURPOSE

This document outlines the procedures for the qualitative or quantitative analysis of ethanol, methanol, acetone, and isopropanol in toxicology specimens using headspace sampling and dual column gas chromatography with flame ionization detection (GC/FID).

## 2 SCOPE

This procedure is used for the qualitative or quantitative analysis of ethanol, methanol, acetone, and isopropanol in toxicology specimens.

## 3 SAFETY

This procedure must be conducted in accordance with the HFSC Health and Safety Manual and Quality Manual. All case specimens should be treated with Universal Blood borne Pathogen Precautions. Appropriate personal protective equipment must be worn during sample and reagent preparation and when handling volatile or caustic chemicals. Material Safety Data Sheets (MSDS) are available in the laboratory.

## 4 REAGENTS

4.1 Ethyl Alcohol (Ethanol, $CH_3CH_2OH$) [64-17-5] (200 proof)
4.2 n-Propanol ($CH_3CH_2CH_2OH$) [71-23-8]
4.3 Isopropanol ($CH_3CH[OH]CH_3$) [67-63-0]
4.4 Acetone ($CH_3COCH_3$) [67-64-1]
4.5 Methanol ($CH_3OH$) [67-56-1]
4.6 Deionized (DI) Water [7732-18-5]
4.7 Blank Blood (Potassium Oxalate and Sodium Fluoride preserved)

## 5 EQUIPMENT

5.1 Instrumentation using a method that is approved and validated for use in the section
5.2 Compressed gas cylinders or equivalent (Helium, Hydrogen, Air, and Nitrogen)
5.3 Vials, caps, stoppers, and crimpers
5.4 Hamilton pipettor-dilutor or equivalent
5.5 Volumetric flasks (50 mL, 100 mL, 500 mL, 1000 mL, etc.)
5.6 Analytical balance
5.7 Homogenizer
5.8 Centrifuge
5.9 Vortex mixer

## 6 MAINTENANCE

A verification run including calibrators and at least 3 replicates of BQC1, BQC2, EQC, and LMQC should be performed following any preventative maintenance and/or major repairs. Verification run must meet acceptance criteria outlined in this procedure. If acceptance criteria are not met, appropriate measures must be taken to rectify the problem. Verification run must be documented in the maintenance log and data kept in a retrievable format in the laboratory.

## 7 CALIBRATION

### 7.1 Ethanol Calibration (0.010 – 0.500 g/100 mL)

7.1.1 Ethanol calibrators must be run with the first batch of case specimens prepared by an individual analyst each day. Subsequent batches prepared by the same analyst during the same work shift must contain controls prepared along with the case specimens.

7.1.2 Purchased (e.g., Cerilliant, Absolute, or equivalent) NIST certified reference ethanol standards or mixed volatile standards that include ethanol are used as calibrators for ethanol calibration. See manufacturer's Certificate of Analysis for storage and expiration information. The following calibrator concentrations are used for ethanol calibration unless otherwise specified in the case record:

Level 1: 0.010 g/100 mL
Level 2: 0.050 g/100 mL
Level 3: 0.100 g/100 mL
Level 4: 0.200 g/100 mL
Level 5: 0.400 g/100 mL
Level 6: 0.500 g/100 mL

7.1.3 Purchased calibrator solutions typically come in vials containing approximately 1 mL of solution. Once opened, the contents of the vial may be transferred to a labeled container, sealed, and stored in the refrigerator. Opened and transferred solutions can be used for ethanol calibration for 1 month.

### 7.2 Methanol/Isopropanol/Acetone (M/I/A) Calibration (0.010 – 0.400 g/100 mL)

7.2.1 If historical calibration is used for M/I/A, it must be re-established or re-verified at least once every six months OR when one of the following occurs:

7.2.1.1 New n-propanol internal standard (I.S.) stock solution is prepared
7.2.1.2 Significant instrument maintenance has been performed
7.2.1.3 Troubleshooting quality issues

7.2.2 In-house prepared, purchased (e.g., Cerilliant, Absolute, or equivalent) NIST certified reference mixed volatile standards, or a combination of the two are used as calibrators to establish the M/I/A calibration. The following calibrator concentrations are used unless otherwise specified in the case record:

Level 1: 0.010 g/100 mL
Level 2: 0.050 g/100 mL
Level 3: 0.100 g/100 mL
Level 4: 0.400 g/100 mL

## 8 INTERNAL STANDARD

### 8.1 n-Propanol Internal Standard Stock Solution - 1.0 g/100 mL

8.1.1 Weigh out 1.0 g of n-propanol in a 100 mL volumetric flask. Bring to volume with DI water. This will give a 1.0% I.S. stock solution (1.0 g/100 mL).
**Storage**: Store refrigerated. **Discard**: 6 months.

8.2 **n-Propanol Internal Standard Working Solution - 0.010 g/100 mL**

8.2.1 Add 10 mL of the 1% I.S. stock solution to a 1000 mL volumetric flask. Bring to volume with DI water. This will give a 0.010% I.S. working solution (0.010 g/100 mL).
**Storage:** Store at room temperature. **Discard**: 9 months.

9 **CONTROLS**

**NOTE:** Calibrators and controls must be prepared from separate lots or sources of certified reference material. Controls may be purchased or prepared in-house. In-house prepared controls shall be verified to establish a target mean before use by replicates of 3 over 4 runs or a minimum of 12 replicates over several runs.

9.1 **Whole Blood Controls:**

9.1.1 Purchased whole blood ethanol controls at low (BQC1) and high (BQC2) concentrations will be used for quantitative ethanol analysis. A target mean shall be established for each new lot of BQC1 and BQC2 by replicates of 3 over 4 runs or a minimum of 12 replicates over several runs.
**Storage:** Store refrigerated. **Discard:** Expiration date, 1 month opened.

9.1.2 If quantitative M/I/A analysis is required, a purchased mixed volatile whole blood control (MQC) must be used in addition to the ethanol only blood controls (BQC1 and BQC2). A target mean for each analyte shall be established for each new lot of MQC by replicates of 3 over 4 runs or a minimum of 12 replicates over several runs.
**Storage:** Store refrigerated. **Discard:** Expiration date, 1 month opened.

9.2 **Low Aqueous Mixed Volatile Control (LMQC) – 0.020 g/100 mL**

9.2.1 Certified reference materials are purchased to make the LMQC. Spike 25.4 µL each of ethanol, methanol, isopropanol, and acetone into a 100 mL volumetric flask filled with approximately 90 mL of DI water. Bring to volume (QS) with DI water. Cap and mix thoroughly. Carefully transfer into containers that are pre-labeled. Tightly cap each container.
**Storage:** Store in freezer. **Discard**: 1 year frozen, 1 month thawed.

9.3 **Aqueous Ethanol Control (EQC) – 0.080 g/100 mL**

9.3.1 The EQC is a purchased quality control. Once open, the contents of the vial may be transferred to a labeled container and tightly capped.
**Storage:** Store refrigerated. **Discard:** Expiration date, 1 month opened.

9.4 **High Aqueous Ethanol Control (HEQC) – 0.400 g/100 mL**

9.4.1 Certified reference material is purchased to make HEQC. Spike 5.1 mL of ethanol into a 100 mL volumetric flask filled with approximately 90 mL of DI water. Bring to volume (QS) with DI water. Cap and mix thoroughly. Carefully transfer into containers that are pre-labeled. Tightly cap each container.
**Storage:** Store in freezer. **Discard**: 1 year frozen, 1 month thawed.

9.5 **Mixed Volatile System Suitability Control (SS) – ~0.010 g/100 mL ethanol, methanol, isopropanol, and acetone**

9.5.1 The system suitability is a qualitative control used to confirm resolution prior to each

calibration. The concentration should be approximately 0.010 g/100 mL of each analyte. This can be a purchased aqueous control or remaining calibration material from the historical calibration.

9.6 **Dilution control (DQC)**

9.6.1 A dilution of the HEQC or other CRM with known concentration is analyzed in the batch, if necessary, to verify dilution results of an alcoholic beverage or other liquid case specimens.

10 **SAMPLE PREPARATION**

10.1 Allow calibrators, controls, and case specimens to come to room temperature prior to sampling.

10.2 Mix all calibrators, controls, and case specimens well prior to sampling by gentle inversion or rocking. Avoid shaking.

10.3 The DI Water Control, consisting of 50 µL deionized (DI) water, is left open for the duration of the sampling process. When all sampling is completed, add internal standard, cap, and crimp tightly.

10.4 Dilutions will be performed with deionized (DI) water prior to sampling.

10.4.1 When alcoholic beverages or other liquids are analyzed, which is infrequent, fluid from the container is diluted appropriately based on suspected alcohol content prior to analysis. From the dilution, a 50 µL aliquot is analyzed.

10.4.2 Example: For a 4% alcoholic beverage, a 1:20 dilution is appropriate.

10.4.3 A DQC, using the same dilution as the case specimen (if possible), must be included in the batch when diluted alcoholic beverages or other liquids are analyzed.

10.4.4 Commonly used dilutions are as follows:

| Dilution | Beverage (µL) | Deionized Water (µL) |
|---|---|---|
| 1:20 | 50 | 950 |
| 1:50 | 20 | 980 |
| 1:100 | 10 | 990 |

10.5 Using the pipettor-dilutor, aliquot 50 µL of the calibrators, controls, and case specimens. The pipettor-dilutor will deliver 500 µL of the I.S. working solution along with the 50 µL sample aliquot into the appropriately labeled headspace vial. Cap and crimp tightly.

Note: Samples must be prepared in the same order they will be analyzed (see sequence below). Between each sample, rinse the pipettor-dilutor tubing with DI-water, at least 2 flushes of the internal standard solution, and wipe tubing.

10.6 All case specimens are analyzed in duplicate. A single aliquot will be taken from each case specimen and recapped. Once all case specimens have been singly prepared for analysis, they will be re-ordered prior to sampling a second time. This can be accomplished by preparing case specimens in reverse order.

10.7 For cases that have multiple items submitted, one specimen from each item will be analyzed. Preference will be given in the following order: grey>lavender>pink>tan> royal blue (if it contains anticoagulant).

10.8 The condition of a case specimen during analysis must be recorded (e.g., normal, thick, clotted, decomposed). If a case specimen is clotted, the specimen must be homogenized using a tissue grinder or equivalent prior to analysis.

10.9 Transfer vials from the sampling rack to the Autosampler using batch sequence.

## 11 SEQUENCE TABLES

11.1 **Example sequence**:

SS
Calibrators (Low to High)
Negative Control (Carryover check)
HEQC
BQC2
EQC
MQC[1]
DQC[2]
Up to 10 samples
BQC1
Up to 10 samples
BQC2
Up to 10 samples
Continue to alternate 10 samples with low and high controls
BQC1*
LMQC*
EQC*
DI Water Control*

[1] Only necessary if quantifying M/I/A.
[2] Only necessary if diluted case specimen(s) are included in batch.
*NOTE: Each sample batch shall end with BQC1, LMQC, EQC, and DI Water Control.

11.2 The order of samples on the autosampler must be verified by a second reviewer, before or after analysis. This must be documented by initialing and dating the sequence.

11.3 Batch size will accommodate all accessioned case specimens or a maximum of 30 case specimens.

## 12 CALCULATION AND ACCEPTANCE CRITERIA

Analyte concentrations are determined by linear regression (y = mx + b) with 1/x weighting, based on the ratio of the peak area of the calibrator divided by the peak area of the internal standard. The calibration curve is calculated by instrument software.

12.1 **Calibrators and Controls (Ethanol and M/I/A)**

12.1.1 For volatile concentrations > 0.050 g/100 mL, obtained values from FID1 A (BAC1) must be within ± 5% of target value.

12.1.2 For volatile concentrations ≤ 0.050 g/100 mL, obtained values from FID1 A (BAC1) must be within ± 10% of target value.

12.1.3 The calibration curve must yield an $R^2$ value of 0.99 or greater.

12.1.4 If a control or calibrator is not within the given range:
The associated control(s) or calibrator(s) will be re-evaluated a second time. If after the second analysis the result remains outside the allowable range, the section Manager or Supervisor will be consulted.

12.1.5 Any case specimens bracketed by a control not within the acceptable range must be re-analyzed.

12.2 **Internal Standard Recovery**

12.2.1 For each batch, the consistency of internal standard must be evaluated.

12.2.2 The internal standard recovery, as indicated by the area counts of the integration, must be within ½ to 2 times the average of the calibrators and controls within the batch.

12.2.3 If an unknown sample is not within this range, the sample may be re-injected once. If it is still not within this range, the sample must be re-aliquotted.

12.3 **Chromatography**

12.3.1 All chromatography must be well resolved from any interfering peaks and symmetric.

12.3.2 Peak symmetry will be evaluated for every peak associated with an analyte of interest. The instrument software will generate and report the peak symmetry. A number between 0.5 and 2 indicates acceptable symmetry.

12.3.3 Peak resolution will be evaluated for every peak associated with an analyte of interest. The instrument software will generate and report the peak to peak valley ratio for peaks that have an apparent shoulder or interfering peak. A number greater than 10 indicates acceptable resolution.

12.3.4 Any samples with unresolved or asymmetric peaks may be re-injected once. If the same condition is found, the sample must be re-aliquotted.

12.4 **Case specimens**

12.4.1 **Ethanol**

12.4.1.1 Results from FID1 A (BAC1) and FID2 B (BAC2) per aliquot must be within ± 5% of each other.

12.4.1.2 If the average, truncated to 3 decimals, of aliquot 1 and aliquot 2 from FID1 A (BAC1) ≤ 0.050 g/100 mL; both aliquot values must be ± 10% of the average.

$$Acceptable\ Range = \frac{Aliquot\ 1\ + Aliquot\ 2}{2}\ x\ 0.9\ to\ \frac{Aliquot\ 1 + Aliquot\ 2}{2}\ x\ 1.1$$

12.4.1.3 If the average, truncated to 3 decimals, of aliquot 1 and aliquot 2 from FID1 A (BAC1) > 0.050 g/100 mL; both aliquot values must be ± 5% of the average.

$$Acceptable\ Range = \frac{Aliquot\ 1 + Aliquot\ 2}{2}\ x\ 0.95\ to\ \frac{Aliquot\ 1 + Aliquot\ 2}{2}\ x\ 1.05$$

12.4.1.4 If a case specimen is not within the given range, that case specimen must be re-analyzed. If after two analyses the duplicate difference for each run exceeds the acceptable range, the lowest obtained blood alcohol value from all four results (FID1 A) will be used. Any further attempts should be investigated and discussed with the section Manager, Supervisor, or Quality Manager.

12.4.2 **M/I/A**

12.4.2.1 These compounds can be reported qualitatively if the calculated concentration is ≥ 0.010 g/100 mL and the retention time is within 2% of that obtained for each analyte of interest in the LMQC.

12.4.2.2 If a quantitative value is needed for M/I/A, positive specimens must be re-analyzed with the inclusion of MQC in the batch and MQC must meet acceptance criteria to report a quantitative result.

12.4.2.3 The values from aliquot 1 and aliquot 2 on FID1 A (BAC1) must be within ± 10% of the average, truncated to 3 decimals, of aliquot 1 and aliquot 2.

$$Acceptable\ Range = \frac{Aliquot\ 1 + Aliquot\ 2}{2}\ x\ 0.9\ to\ \frac{Aliquot\ 1 + Aliquot\ 2}{2}\ x\ 1.1$$

## 13 MEASUREMENT UNCERTAINTY (MU)

This is a statistical calculation of all known variables that contribute to the inherent variance of the overall result at a desired confidence level. For this procedure, the confidence level used is 99.73%, k=3. The measurement uncertainty will be evaluated yearly.

13.1 Uncertainty components that contribute to the calculated measurement uncertainty (MU%) include:

13.1.1 Measurement Reproducibility – accounts for the control with the largest Percent Relative Standard Deviation or %RSD based on historical data.

13.1.2 CRM or Certified Reference Material Uncertainty – accounts for the CRM used as a calibrator or control with the largest reported uncertainty.

13.1.3 Pipettor Dilutor – accounts for the variability of the two syringes used by the Hamilton pipettor dilutor for creating samples for analysis. Values are obtained from yearly calibration certificates.

13.1.4 Duplicates – accounts for the maximum allowed difference between analytically-obtained values of duplicate case samples.

13.2 The Alcohol Measurement Uncertainty Spreadsheet is available in the laboratory in a retrievable format.

14 **REPORTING**

14.1 **Ethanol and M/I/A**

14.1.1 Reported concentration will be the average of the results obtained from aliquot 1 and aliquot 2 on FID1 A (BAC1 column), truncated to three decimal places. FID2 B (BAC2 column) is used as a semi-quantitative confirmation only.

14.1.2 Reported measurement uncertainty (MU) will be calculated using the following formula and truncated to three decimal places:

Reported MU = ± (Reported concentration x MU%)

14.2 **Reporting Statements**

14.2.1 Blood specimen results are reported in “grams per 100 milliliters of blood.”

14.2.2 The reported MU must be included on each report using the statement “The measurement uncertainty associated with this value is ± x.xxx grams per 100 milliliters” or an equivalent statement.

14.2.3 For volatiles in alcoholic beverages or other liquid specimens, results are reported in “grams of ethanol per 100 milliliters of liquid.”

14.2.4 For serum/plasma specimens, the report should state “Item x.x was found to contain x.xxx grams of ethanol per 100 milliliters of serum/plasma. The equivalent whole blood concentration was calculated to be x.xxx - x.xxx grams of ethanol per 100 milliliters of blood using a conversion factor range of 1.15 to 1.2” or equivalent statement.

14.2.5 For specimens that required homogenization, the report should state “This sample was homogenized prior to analysis” or an equivalent statement.

14.2.6 For alcoholic beverages or liquid specimens, the results are reported in "grams per 100 milliliters of liquid.”

14.2.7 If no volatiles are detected, or if results are below the lowest calibrator (0.010 g/100 mL), the report should state “no alcohol detected” or an equivalent statement.

14.2.8 If there is insufficient sample, the report should state “Insufficient sample for analysis” or equivalent statement.

14.2.9 If the sample is unsuitable for analysis, the report should state “Sample unsuitable for analysis” or equivalent statement.

# Quantitative Method Validation

## 1 PURPOSE

This procedure is intended to define the minimum parameters and sets of experiments to validate a quantitative method.

## 2 SCOPE

This procedure applies to all quantitative bio-analytical methods.

Method validation is required to verify the performance parameters of a method are fit for purpose. Validation is required after the following events occur:

2.1 Development of a new method

2.2 Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

2.3 Transfer of a validated method to a new instrument

Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. Determination of validation parameters which must be re-evaluated will be dependent upon the changes made to the method.

## 3 VALIDATION PARAMETERS

The following validation parameters are recommended by the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. Please refer to the document published May 20, 2013 for detailed information on each parameter.

3.1 Calibration Model/Linearity

3.2 Limit of Detection (LOD)

3.3 Limit of Quantification (LOQ)

3.4 Bias and Precision

3.5 Carryover

3.6 Interference Studies

3.7 Matrix Matching (if applicable, not included in SWGTOX guidelines)

3.8 Dilution integrity (if applicable)

3.9 Stability (if applicable)

3.10 Ionization suppression/enhancement (if applicable)*

*Note: At this time HFSC does not employ Liquid Chromatography-Tandem Mass spectrometry for any analysis. Ionization suppression/enhancement is not applicable to GCMS testing.

## 4 VALIDATION EXPERIMENTS

### 4.1 Calibration Model/Linearity

The most appropriate calibration model should be determined during method development. For methods using a detector that is theoretically linear over the covered concentration range, it is recommended that initially a straight line concentration-response model be adopted using weighted linear regression with inverse concentration weighting to estimate slope and

intercept. Other procedures may be probed, preferably in the following sequence:

Straight line fit using weighted linear regression with inverse concentration-squared weights
Straight line fit forced through the origin for single point calibrations only
Quadratic fit with inverse concentration using a weighted 2nd order polynomial

There are three components to validation of the calibration model/linearity – verification of weighting scheme, validation of linearity across the analytical measurement range and validation of calibration.

4.1.1 **Procedure**

Once the best calibration model has been determined the following experiment is to be performed to evaluate the effectiveness of the model:

4.1.1.1 Prepare a series of standards covering the concentration range of interest with typically no more than a 5-fold increase between sequential calibrators. At least six (nonzero) concentration levels should be used.

4.1.1.2 A minimum of 5 replicates per concentration is required. The replicates shall be in separate runs. The origin shall not be included as a calibration point.

4.1.2 **Data Analysis**

4.1.2.1 Use data from all five runs to produce combined calibration curves with no weighting, 1/x weighting and $1/x^2$ weighting and process data calibration data using all three calibration curves. This can be done by exporting the data and using a validated excel worksheet designed for this purpose.

4.1.2.1.1 These combined curves will be used to verify the weighting scheme.

4.1.2.2 Using the data from the appropriate weighting scheme performs an unweighted linear regression of $C_{nominal}$ v. $C_{calculated}$ where $C_{nominal}$ is nominal (labeled or established) concentration of calibrator and $C_{calculated}$ is concentration obtained from calibration curve.

4.1.2.2.1 These data will be used to evaluate the linearity of the analytical measurement range.

4.1.2.3 Process each individual run using the selected weighting scheme.

4.1.2.3.1 These data will be used to validate the calibration range.

4.1.2.4 If fewer calibrators will be used to analyze case samples than were used in validation, reprocess all data using only the calibrators selected to be used during case analysis. Perform an unweighted linear regression of $C_{valcal}$ v. $C_{casecal}$ where $C_{valcal}$ is the calculated concentrations of calibrators and controls using all calibrators included in the validation experiments and $C_{casecal}$ is the calculated concentrations of calibrators and controls using the calibrators that will be used during analysis of case samples.

4.1.3 **Acceptance Criteria**

4.1.3.1 **Verification of weighting scheme**

4.1.3.1.1 Correct weighting must be verified by evaluating the $\Sigma|\%RE|$ for at least unweighted, 1/x and $1/x^2$. The least complex weighting scheme that minimizes $\Sigma|\%RE|$ should be used.

$$\%RE = \frac{C_{calculated} - C_{nominal}}{C_{nominal}} \times 100$$

4.1.3.2 **Validation of linearity**

4.1.3.2.1 Visual examination of combined calibration curve with appropriate weighting must verify linearity across analytical range

4.1.3.2.1.1 The linear regression of $C_{nominal}$ vs $C_{calculated}$ must meet the following requirements:

4.1.3.2.1.1.1 95% CI of slope must include 1

4.1.3.2.1.1.2 95% CI of intercept must include 0

4.1.3.3 **Validation of calibration**

4.1.3.3.1 Visual examination of calibration curve must verify linearity across analytical range

4.1.3.3.1.1 %RE of each calibrator, as determined by the weighted linear regression of the individual run, must be ≤ ±20%

4.1.3.4 **Validation of casework calibration**

4.1.3.4.1 The linear regression of $C_{valcal}$ vs $C_{casecal}$ must meet the following requirements:

4.1.3.4.1.1 95% CI of slope must include 1

4.1.3.4.1.2 95% CI of intercept must include 0

**NOTE: All remaining validation experiments should be evaluated using results from the data analysis which includes only the calibrators which are to be used in the analysis of case samples.**

4.2 **Limit of Detection (LOD)**

4.2.1 **Procedure**

Analyze a minimum of three different sources of blank matrix fortified at decreasing concentrations in duplicate.

4.2.2 **Data Analysis**

4.2.2.1 Visually inspect the chromatograms to evaluate the retention time, peak shape, mass spectral ion ratios and any other criteria used to identify the analyte of interest.

4.2.2.2 Use the instrument software to evaluate the signal to noise ratio in the blanks and the LOD samples.

4.2.3 **Acceptance Criteria**

The LOD is the lowest concentration that:

4.2.3.1 Yields a reproducible instrument response greater than or equal to three times the noise level of the background signal from the negative samples.

4.2.3.2 Achieves acceptable predefined detection and identification criteria (e.g., retention time, peak shape, mass spectral ion ratios).

4.3 **Limit of Quantitation (LOQ)**

4.3.1 **Procedure**

4.3.1.1 Prepare a pooled fortified matrix sample at the same concentration as the lowest calibrator.

4.3.1.2 Analyze a minimum of three replicates a run in five different runs.

4.3.2 **Data analysis**

4.3.2.1 Use the established calibration curve to quantify the analyte of interest.

4.3.2.2 Calculate the bias

$$Bias = \frac{Average\ of\ calculated\ concentrations - Nominal\ concentration}{Nominal\ Concentration}\ x\ 100$$

4.3.2.3 Calculate the within-run and between-run Precision

$$Within - Run\ Imprecision = \frac{Standard\ deviation\ of\ single\ run}{Average\ of\ single\ run}\ x\ 100$$

$$Between - Run\ Imprecision = \frac{Standard\ deviation\ of\ all\ runs}{Average\ of\ all\ runs}\ x\ 100$$

4.3.3 **Acceptance Criteria**

4.3.3.1 Bias: %Bias ≤ 25%

4.3.3.2 Within-Run Precision: % CV ≤ 25%

4.3.3.3 Between-Run Precision: % CV ≤ 25%

4.4 **Bias and Precision**

4.4.1 **Procedure**

4.4.1.1 Prepare pooled fortified matrix samples using a minimum of three replicates per run at three control concentrations (low, medium, and high).

4.4.1.2 Analyze a minimum of three replicates per run in five different runs.

4.4.2 **Data Analysis**

4.4.2.1 Use the established calibration curve to calculate the concentration of the analyte of interest.

4.4.2.2 Calculate bias, within-run and between-run precision.

4.4.3 **Acceptance Criteria**

4.4.3.1 Bias: %Bias ≤ 20%

4.4.3.2 Within-Run Precision: % CV ≤ 20%

4.4.3.3 Between-Run Precision: % CV ≤ 20%

4.5 **Carryover**

4.5.1 **Procedure**

4.5.1.1 Analysis of blank matrix samples (with IS) following the high calibrator in each validation run

4.5.2 **Data Analysis**

4.5.2.1 Use the established calibration curve to calculate the concentration of the analyte of interest.

4.5.2 **Acceptance Criteria**

4.5.2.1 Quantitative result must be ≤25% of LOQ

4.6 **Interference Studies**

4.6.1 **Procedure**

4.6.1.1 Matrix interference – Analyze a minimum of 10 different sources of blank matrix (without IS).

4.6.1.1 Interference from stable-isotope internal standards – Analyze a blank matrix fortified with IS but NO analyte of interest in each validation run.

4.6.1.2 Interference from commonly encountered exogenous analytes – Analyze blank matrix fortified with analytes of interest at the low control concentration and potential interferences at high therapeutic or lethal concentrations.

4.6.2 **Data analysis**

4.6.2.1 Matrix interference – Evaluate response of any peak at the retention time of the analyte of interest.

4.6.2.2 Interference from stable-isotope internal standards – Evaluate response of any peak at the retention time of the analyte of interest.

4.6.2.3 Interference from commonly encountered exogenous analytes –Use the established calibration curve to calculate the concentration of the analyte of interest.

4.6.3 **Acceptance Criteria**

4.6.3.1 Matrix interference – Response of blank matrix must be ≤ 10% of the average response of LOQ.

4.6.3.2 Interference from stable-isotope internal standards – Response of blank matrix must be ≤ 10% of the average response of LOQ.

4.6.3.3 Interference from commonly encountered exogenous analytes - Concentration of analytes of interest must meet acceptance criteria of the method.

4.7 **Matrix Matching**

If matrices other than that used to create the calibration will be analyzed, the ability of the method to accurately quantify analytes in that matrix must be evaluated. Example: a method uses blood calibrators but will be used to analyze serum samples.

4.7.1 **Procedure**

4.7.1.1 Perform bias and precision studies in each matrix that will be analyzed using the method.

4.7.1.2 Ideally multiple sources of each matrix should be used for these studies.

4.7.2 **Data Analysis**

4.7.2.1 Use the established calibration curve to calculate the concentration of the analyte of interest.

4.7.2.2 Calculate bias, within-run and between-run precision.

4.7.3 **Acceptance Criteria**

4.7.3.1 Bias: %Bias ≤ 20%

4.7.3.2 Within-Run Precision: % CV ≤ 20%

4.7.3.3 Between-Run Precision: % CV ≤ 20%

4.8 **Dilution integrity (if applicable)**

4.8.1 **Procedure**

4.8.1.1 Dilute the high control with blank matrix (evaluate dilution ratios that may be used on case specimens). Common dilutions are 2x (1 part sample + 1 part diluent), 5x (1 part sample + 4 parts diluent), and 10x (1 part sample + 9 parts diluent).

4.8.1.2 Analyze a minimum of two replicates of each dilution per run for at least three runs.

4.8.2 **Data Analysis**

4.8.2.1 Calculate bias and precision as described above.

4.8.2.2 Note: The average control concentrations from the bias and precision experiments can be used as the target for evaluating dilution integrity.

4.8.3 **Acceptance Criteria**

4.8.3.1 All diluted samples must quantify within control acceptance criteria (typically 20%) of the average value obtained in the Bias and Precision studies.

4.9 **Stability**

4.9.1 **Procedure**

4.9.1.1 **Storage stability** – A literature search should be performed to determine if storage stability for the analyte of interest has been reported. If no information can be found then follow the procedure below.

4.9.1.1.1 Determine what conditions will be evaluated. At minimum, stability should be investigated in gray-top tubes stored at room temperature, refrigerated temperature and frozen. If other types are expected to be received, they should also be investigated.

NOTE: If analytes are determined to be unstable at all storage conditions an investigation of the effects of light protection should be considered.

4.9.1.1.2 Prepare enough control material at low and high control concentrations to complete entire stability experiment.

4.9.1.1.3 Store specimens at determined temperatures.

4.9.1.1.4 Analyze samples in triplicate after storage for 0, 7, 14, and 30 days and 6 months. NOTE: It is permissible to use Day 1 validation QC results as "Day 0" for stability purposes

4.9.1.2 **In-Process stability** – if it is expected that a break may need to take place during sample preparation, the stability of the analyte over that time period should be evaluated.

4.9.1.2.1 Determine the point in the procedure at which a break may occur and how long the break may be. In the case of procedures where solid phase or liquid extractions are performed, the studied step should be the final extraction or elution solvent.

4.9.1.2.2 Prepare a calibration curve and controls according to the normal procedure.

4.9.1.2.3 Prepare a second set of controls, in duplicate, but delay completion of preparation at the predetermined step and for the correct time.

4.9.1.3 **Auto-sampler stability** – Both the analyte(s) and the internal standard in processed QC samples will be tested for stability. This will demonstrate that extracts are stable while in the auto-sampler awaiting analysis. In addition, determination of stability beyond 12 hours will allow for the re-injection of samples in the event of instrument malfunction.

4.9.1.3.1 Determine the maximum residence time of the extracts in the auto-sampler to be tested. Include the time a run may sit unanalyzed over a weekend in the auto-sampler due to an instrument malfunction. A different approach may be necessary for unstable molecules.

4.9.1.3.2 Set aside a validation run that includes calibrators, and at least 2 low and 2 high controls to be re-injected. These samples will be added to another validation run with a freshly prepared calibrator.

4.9.1.3.3 Prepare a calibration curve along with at least 4 low and 4 high controls.

4.9.1.3.4 Inject the calibration curve only and one set (at least 2 low and 2 high) controls.

4.9.1.3.5 Store the remaining vials in the auto-sampler for at least 12 hours. The interval chosen should represent the longest period that the processed extracts will remain at auto-sampler conditions during the course of an analytical run.

4.9.1.3.6 At the end of the predetermined time period inject all controls (half will be unanalyzed, half will be re-injects)

4.9.2 **Data Analysis**

4.9.2.1 Use the established calibration curve to calculate the concentration of the analyte of interest.

4.9.3 **Acceptance Criteria**

4.9.3.1 All storage stability samples must quantify within control acceptance criteria (typically 20%) of the method compared to the average concentration on Day 0.

4.9.3.2 All in-process and auto-sampler stability samples must quantify within control acceptance criteria of the average value obtained in the Bias and Precision studies.

# Validation of Reportable Qualitative Analytical Methods

1 **PURPOSE**

This procedure is intended to define the minimum parameters and sets of experiments to validate a reportable qualitative method.

2 **SCOPE**

This procedure applies to all qualitative bio-analytical methods which produce reportable results.

Method validation is required to verify the performance parameters of a method are fit for purpose. Validation is required after the following events occur:

2.1 Development of a new method

2.2 Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

2.3 Transfer of a validated method to a new instrument

Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. Determination of validation parameters which must be re-evaluated will be dependent upon the changes made to the method.

3 **Definitions**

Cutoff calibrator – matrix sample fortified with the analyte of interest at the reporting limit of the assay

Qualitative Negative Control – matrix sample fortified with the analyte of interest at a concentration no less than 50% of the cutoff calibrator.

Qualitative Positive Control – matrix sample fortified with the analyte of interest at a concentration no more than 200% of the cutoff calibrator

Blind samples – matrix matched samples containing the analyte of interest above and below the cutoff concentration

True Positive (TP) – sample containing the analyte of interest above the cutoff concentration that gives a positive result

True Negative (TN) – sample containing the analyte of interest below the cutoff concentration that gives a negative result

False Positive (FP) – sample containing the analyte of interest below the cutoff concentration that gives a positive result

False Negative (FN) – sample containing the analyte of interest above the cutoff concentration that gives a negative result

## 4 VALIDATION PARAMETERS

The following validation parameters are recommended by the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. Please refer to the document published May 20, 2013 for detailed information on each parameter.

4.1 Precision around reporting limit (RL)
4.2 Sensitivity/Specificity
4.3 Interference Studies
4.4 Stability (if applicable)
4.5 Ionization suppression/enhancement (if applicable)*

*Note: At this time HFSC does not employ Liquid Chromatography-Tandem Mass spectrometry for any analysis. Ionization suppression/enhancement is not applicable to GCMS testing.

## 5 VALIDATION EXPERIMENTS

### 5.1 Precision around reporting limit (RL)

#### 5.1.1 Procedure

5.1.1.1 Prepare a pooled fortified matrix sample at the negative control; cutoff calibrator, and positive control concentrations.

5.1.1.2 Analyze 5 samples a run at each concentration over a minimum of 5 runs (25 samples minimum at each concentration)

#### 5.1.2 Data analysis

5.1.2.1 Determine if each sample is positive or negative

#### 5.1.3 Acceptance Criteria

5.1.3.1 At least 22 (90%) of the negative controls must be negative

5.1.3.2 At least 22 (90%) of the positive controls must be positive

5.1.3.3 8-16 (35-65%) of the cutoff calibrators must be positive

### 5.2 Sensitivity/Specificity

#### 5.2.1 Procedure

Prepare a total of 40 blind specimens. These specimens can be discarded patient specimens know to be positive or negative for PCP or fortified samples. 20 of the samples should have concentrations of 0-75% of the cutoff calibrator concentration and 20 should have the analyte of interest at concentrations of ≥125% of the cutoff concentration. The expected result of these samples should be blinded to the analyst performing the validation.

#### 5.2.2 Data Analysis

5.2.2.1 Determine if each sample is positive or negative

5.2.2.2 Calculate Sensitivity and Specificity

$$\text{Sensitivity} = \frac{\text{TP}}{\text{TP}+\text{FN}} \text{ x } 100$$

$$\text{Specificity} = \frac{TN}{TN+FP} \text{ x } 100$$

5.2.3 **Acceptance Criteria**

5.2.3.1 Sensitivity ≥ 90%

5.2.3.2 Specificity ≥ 90%

5.3 **Interference Studies**

5.3.1 **Procedure**

5.3.1.1 Matrix interference – Analyze a minimum of 10 different sources of blank matrix (without IS).

5.3.1.2 Interference from stable-isotope internal standards – Analyze a blank matrix fortified with IS but NO analyte of interest in each validation run.

5.3.1.3 Interference from commonly encountered exogenous analytes – Analyze blank matrix fortified with analytes of interest at the negative control concentration and potential interferences at high therapeutic or lethal concentrations.

5.3.2 **Data analysis**

5.3.2.1 Matrix interference – determine if sample is positive or negative

5.3.2.2 Interference from stable-isotope internal standards – determine if sample is positive or negative

5.3.2.3 Interference from commonly encountered exogenous analytes –determine if sample is positive or negative

5.3.3 **Acceptance Criteria**

All samples must test negative.

5.4 **Stability**

5.4.1 **Procedure**

5.4.1.1 **Storage stability** – A literature search should be performed to determine if storage stability for the analyte of interest has been reported. If no information can be found then follow the procedure below.

Determine what conditions will be evaluated. At minimum, stability should be investigated in plastic urine cups stored at room temperature, refrigerated temperature and frozen. If other types are expected to be received, they should also be investigated.

NOTE: If analytes are determined to be unstable at all storage conditions an investigation of the effects of light protection should be considered.

5.4.1.1.1 Prepare enough positive control material to complete entire stability experiment

5.4.1.1.2 Store specimens at determined temperatures

5.4.1.1.3 Analyze samples in triplicate after storage for 0, 7, 14, and 30 days and 6 months.

NOTE: It is permissible to use Day 1 validation QC results as “Day 0” for stability purposes

5.4.1.2 **In-Process stability** – if it is expected that a break may need to take place during sample preparation, the stability of the analyte over that time period should be evaluated.

5.4.1.2.1 Determine the point in the procedure at which a break may occur and how long the break may be. In the case of procedures where solid phase or liquid extractions are performed, the studied step should be the final extraction or elution solvent.

5.4.1.2.2 Prepare the cutoff calibrator, negative control and positive control according to the SOP

5.4.1.2.3 Prepare a second set of positive controls, in duplicate, but delay completion of preparation at the predetermined step and for the correct time.

5.4.1.3 **Auto-sampler stability** – Both the analyte(s) and the internal standard in processed QC samples will be tested for stability. This will demonstrate that extracts are stable while in the auto-sampler awaiting analysis. In addition, determination of stability beyond 12 hours will allow for the re-injection of samples in the event of instrument malfunction.

5.4.1.4 Determine the maximum residence time of the extracts in the auto-sampler to be tested. Include the time a run may sit unanalyzed over a weekend in the auto-sampler due to an instrument malfunction. A different approach may be necessary for unstable molecules.

5.4.1.5 Prepare a cutoff calibrator curve along with at least 4 positive controls and 1 negative control

5.4.1.6 Inject the calibration curve, negative control and one set (at least 2) positive controls.

5.4.1.7 Store the remaining vials in the auto-sampler for at least 12 hours. The interval chosen should represent the longest period that the processed extracts will remain at auto-sampler conditions during the course of an analytical run.

5.4.1.8 At the end of the predetermined time period inject all controls (half will be unanalyzed, half will be re-injects)

5.4.2 **Data Analysis**

Determine if samples are positive or negative

5.4.3 **Acceptance Criteria**

All positive controls must remain positive

# Validation of Manufactured Kits used for Screening Analysis

## 1 PURPOSE

This SOP is intended to define a series of experiments that are relevant elements of a qualitative immunoassay method validation.

## 2 SCOPE

This procedure applies to immunoassay based methods using commercially available kits.

Method validation is required to verify the performance parameters of a method are fit for purpose. Validation is required after the following events occur:

2.1 Development of a new method

2.2 Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

2.3 Transfer of a validated method to a new instrument

Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. Determination of validation parameters which must be re-evaluated will be dependent upon the changes made to the method.

## 3 VALIDATION PARAMETERS

The following validation parameters are recommended by the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. Please refer to the document published May 20, 2013 for detailed information on each parameter.

3.1 Limit of Detection (LOD)

3.2 Precision at the decision point

3.3 Cross –reactivity (if applicable) – Note: cross-reactivity data is generally provided with commercially available immunoassays thus evaluation of cross-reactivity is generally not needed for immunoassay

3.4 Dilution integrity (if applicable)

3.5 Stability (if applicable) – Note: stability is generally evaluated during the validation of confirmation assays and thus is not needed for immunoassay validation

## 4 VALIDATION EXPERIMENTS

### 4.1 Limit of Detection (LOD)

Typically the LOD of immunoassay based methods is defined as the decision point concentration.

### 4.2 Precision at the decision point Procedure

#### 4.2.1 Procedure

4.2.1.1 Negative (no more than 50% below decision point) and positive controls (no more than 50% above decision point) are prepared and tested for each qualitative method validation. In addition a material of known concentration near the cutoff limit is tested.

4.2.1.2 Perform a daily assay testing each of the three materials (negative, positive and near cutoff) over a period of 5 days, with five replicates per day. Thus, 25 data points are generated for each level. The same lot of reagents and cutoff material is used during the test period.

4.2.2 **Data Analysis**

4.2.2.1 Determine if each sample is positive or negative using the average OD of the 5 replicate cutoff calibrator

4.2.3 **Acceptance Criteria**

4.2.3.1 Expected results for the negative and positive controls

4.2.4 **Monitored data:**

4.2.4.1 These data are calculated as part of method validation to identify any potential issues which may be encountered during routine use. These are not criteria for acceptance or rejection of the immunoassay validation.

4.2.4.1.1 Overall CV of the optical density < 20% at each of the three levels.

4.2.4.1.2 Mean ± 2 standard deviations of the optical density should not overlap the decision point

# Appendix 1. Abbreviations

| | |
|---|---|
| = | equal |
| +/- or ± | plus or minus |
| < | less than |
| > | greater than |
| ≤ | less than or equal to |
| ≥ | greater than or equal to |
| 6-AM or 6-MAM | 6-acetyl morphine or 6-monoacetyl morphine |
| ADA | assistant district attorney |
| ALC | test for the presence of alcohol |
| ALP | alprazolam |
| BAN | basic, acid, neutral drug screen |
| BCR | blood collection report located on inner plastic box |
| BE or BZE | benzoylecgonine |
| BH | biohazard |
| BZ or BENZO | benzodiazepines |
| c or c/ | containing/ |
| C/M | carisoprodol/meprobamate |
| CNS | Central Nervous System |
| COC | cocaine |
| CRM | certified reference material |
| DI | deionized |
| DOB | date of Birth |
| DQC | dilution control |
| DUID | driving under the influence of drugs |
| DWI | driving while intoxicated |
| EIA | enzyme Immunoassay |
| EME | ecgonine methyl ester |
| ENV | envelope |
| EQC | aqueous ethanol control |
| EtAc | ethyl acetate |
| EtOH | ethanol (ethyl alcohol) |
| FN | false negative |
| FP | false positive |
| g | grams |
| GC | gas chromatograph |
| GC/FID | gas chromatograph/flame ionization detector |
| GC/MS | gas chromatograph/mass spectrometer |
| $H_2O$ | water |
| HEQC | high aqueous ethanol control |
| HHC | higher than the highest calibrator |
| hr | hour |
| hrs | hours |
| IB | inner box |
| Incl/ | including |

| | |
|---|---|
| I.S. or IS | internal standard |
| KETO | keto-opioids |
| kg | kilogram |
| L | liter |
| lb | pound |
| LIMS | Laboratory Information Management System |
| LLC | lower than the lowest calibrator |
| LLE | liquid-liquid extraction |
| LMQC | low aqueous mixed volatile control |
| LOD | limit of detection |
| LOQ | limit of quantitation |
| METH | methamphetamine |
| MeOH | methanol (methyl alcohol) |
| mcg, µg, or ug | microgram |
| mg | milligrams |
| min | minute |
| mins | minutes |
| MS | mass spectrometer |
| MU | measurement uncertainty |
| N/A, NA, or na | not applicable |
| Neg | negative |
| NF | not found |
| ng | nanogram |
| OB | outer box |
| OFC or Ofc | officer |
| OPICOC | opiates/cocaine |
| oz | ounce |
| PCP | phencyclidine |
| Pkg | package |
| Pos | positive |
| QC | quality control |
| QNS | quantity not sufficient |
| QS | quantum satis (bring to volume) |
| RL | reporting limit |
| RRT | relative retention time |
| RT | retention time |
| SPE | solid phase extraction |
| SS | mixed volatile system suitability control |
| STIM | stimulants |
| THC | tetrahydrocannabinol |
| THCA | tetrahydrocannabinol carboxylic acid |
| TN | true negative |
| TP | true positive |
| UNK or unk | unknown |
| w/ | with |
| w/out | without |